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FACTORS RELATING TO THE QUANTITATIVE ESTIMATION OF LOW LEVELS OF DIPHTHERIA ANTITOXIN BY THE RABBIT SKIN TEST[1]

BY L. GREENBERG[2] AND J. GIBBARD[3]

Abstract

A simple method for the quantitative determination of small amounts of diphtheria antitoxin is presented. Toxin of known strength is mixed with various dilutions of serum. These are held at room temperature for two hours and 0.1 cc. of each dilution is injected intracutaneously into the back of a suitably prepared rabbit. The resulting skin reactions are compared with reactions obtained with control mixtures, containing known amounts of antitoxin, injected at the same time. Between 200 and 300 tests may be performed on the back of a single rabbit.

Introduction

The classical method of Ehrlich for measuring diphtheria antitoxin is the standard procedure in all countries. This method, however, is only suitable for measuring solutions containing one or more units of antitoxin per cc. For the measurement of small amounts of antitoxin, namely fractions of one unit such as may be found in human sera, intracutaneous tests on guinea pigs and rabbits are used.

In all tests of this type the principles involved are the same. A known amount of toxin is mixed with varying dilutions of antitoxins. The mixtures are held for varying lengths of time and then injected into animals. The degree of the resulting reaction indicates the strength of the antitoxin. For an Ehrlich test, the test dose of toxin is the L+ dose, and this is defined as that amount of the toxin which, when mixed with one unit of antitoxin and injected into a normal guinea pig weighing 250 gm., will kill the guinea pig on the fourth day after the injection. For an intradermal test, the dose of toxin will be that amount of toxin, which, when mixed with a known amount of antitoxin, will upon subsequent intradermal injection into the back of a rabbit or guinea pig cause a definite area of erythema. The known strength of antitoxin used can be varied for the test at hand but is usually 1/250 unit or less per cc.

[1] *Manuscript received October 6, 1948.*

Contribution from the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa.

[2] *Bacteriologist.*

[3] *Chief.*

The method was originally devised with guinea pigs (4, 6) following Römers observation that diphtheria toxin produced a specific and characteristic lesion when injected experimentally into these animals.

The test was modified later for use with rabbits by Fraser (2, 3) and Jensen (5). The procedures described below are details of the method in current use at the Laboratory of Hygiene and are designed to test at a level of 1/250 unit of antitoxin. Although the method is not original it is presented because of its importance to both clinical and control laboratories and because of the number of requests by laboratory workers interested in this technique. Further, workers on this continent do not generally appreciate the large number of tests (over 300) that can be placed on the back of one animal.

General Procedure

I. *The Ingredients*

(*a*) *Toxin*

Only well stabilized toxins should be used. The toxin in use at the Laboratory of Hygiene was stabilized by dilution in a borate buffer* gelatin solution (pH 7.6), the method recommended by Moloney and Taylor (7, 8). A freshly prepared lot of diphtheria toxin was diluted to contain 2 L+ doses per cc. Experimentation has shown the intradermal test dose of toxin to be 1/250 of an L+ dose, contained in 1.0 cc. There has been no detectable change in potency over a period of eight years.

(*b*) *The Antitoxin*

An international standard reference antitoxin is used as a control. It is used in three strengths, 1/125, 1/250, and 1/500 unit per cc.

(*c*) *The Diluent*

Borate buffer saline (pH 8.0) is used as the diluent.

(*d*) *The Test Animal*

Rabbits are used as the test animal. Prior to use, the back and flanks of the animals are closely clipped with suitable clippers. The backs are then marked off into approximately 2 cm. squares by means of a wooden applicator using a saturated solution of picric acid as the marking fluid.

** Adapted from Palitzsch's borax–boric acid mixture (1) the formula used for the preparation of this solution was:—*

(*a*)	*Sodium borate*	—	*13.1 gm.*
	Distilled water	—	*1 liter*
(*b*)	*Boric acid*	—	*12.4 gm.*
	NaCl	—	*2.9 gm.*
	Distilled water	—	*1 liter*
(*c*)	*Solution (a)*	—	*250 cc.*
	Solution (b)	—	*750 cc.*

Adjust to pH 7.6 using Solutions (a) and (b)

Dilute Solution (c) 1:4 using as diluent a 0.85% saline solution containing 0.02% gelatin. Readjust pH to 7.6 using Solutions (a) and (b).

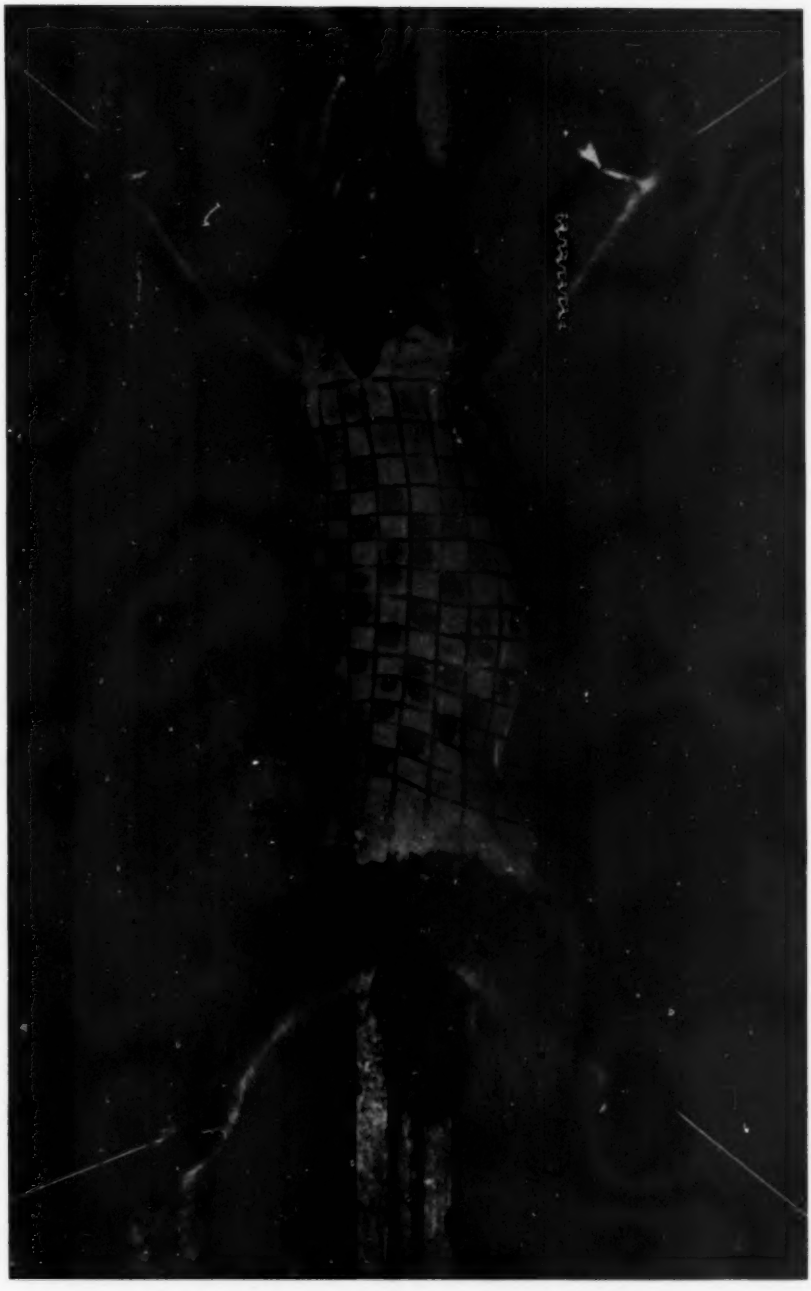

FIG. 1. *A typical rabbit skin test four days after inoculation.*

II. *Technique of Test*

The test dose of toxin is mixed with the same volume of dilutions of the solution to be tested, e.g., undiluted, 1 : 2 dilution, 1 : 4 dilution, etc. The mixtures are held at room temperature for two hours and then 0.1 cc. of each mixture is injected intracutaneously into designated squares on the back of the rabbit prepared as above. To serve as controls for the toxin and also to act as sources of comparison, three control mixtures containing 1/125, 1/250, and 1/500 unit of known antitoxin per cc. respectively are prepared at the same time as the above and injected intracutaneously into several designated regions on the back of the same animal. Three days later the individual skin reactions are measured and recorded. The strength of the unknown solution is measured by direct comparison with the known antitoxin controls. In a properly controlled test, the mixture containing 1/125 unit of antitoxin should leave no visible reaction, the mixture containing 1/250 unit should result in a mild erythema of between 4 to 8 mm. and the mixture with 1/500 unit should give a slightly heavier erythema of between 8 to 12 mm. The test dose of toxin, when injected alone, should cause an erythematous area of 10 to 15 mm. with some necrosis. The number of squares on the back of an animal will be influenced by two factors—the strength of the test toxin used, and the size of the rabbit. Jensen, testing at the 1/3000 antitoxin level, puts over 300 squares on one rabbit. At the Laboratory of Hygiene, where the work is generally with a level of 1/250 and 1/500 antitoxin units, up to 250 squares can be inoculated on one rabbit. The accompanying photograph illustrates a typical test on a rabbit four days after the inoculation.

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DIAGNOSTIC USE OF TETRAETHYLAMMONIUM CHLORIDE (ETAMON) IN OCCLUSIVE ARTERIAL DISEASE OF THE EXTREMITIES[1]

By J. Farr and J. Doupe

Abstract

Using the temperature of the digits to measure vasodilation, the dilating effect of tetraethylammonium chloride (Etamon) was compared to that of thermal stimulation. It was found in three normal subjects and 11 patients that doses of 300 to 500 mgm. administered intravenously never induced a higher skin temperature than did body heating and the mean maximum temperature was 5° to 6° C. lower. Because of the hypotensive effect of the drug higher doses were not used. It was concluded that Etamon was not a reliable agent for testing the vasodilatation potential of the peripheral circulation.

Introduction

In 1945 Acheson and Moe (1) described the paralytic effect of the tetraethylammonium salts upon the ganglia of the autonomic system and in later reports they and others indicated that tetraethylammonium chloride, i.e. Etamon, could be used to determine the amount of vasodilatation possible in patients with organic arterial disease of the extremities. In 1947 Coller *et al.* (3) reported further results and claimed that it was superior to any other method of vasodilatation. However, DeBakey (4) in discussing their paper stated that he had not formed a favorable opinion of the test and Boyd *et al.* (2), using the bromide salt, have contributed evidence supporting this view.

The present investigation was undertaken because of the importance of establishing whether or not such a simple procedure as an intravenous injection could replace the established methods that assist in the evaluation of the degree of organic arterial obstruction in an extremity. Reflex vasodilatation and sympathetic block are the two methods most commonly employed. Because the latter is to a great extent dependent upon the skill of the operator and therefore subject to technical error, reflex vasodilatation was used as the standard to which the effects of Etamon were compared.

Methods

Skin temperatures were used as an index of vasodilatation. They were recorded by means of copper–constantan (gauge 34) couples in contact with the pads of the exposed digits and held in place by narrow strips of adhesive tape on the dorsum of the second phalanx. Temperatures were read to 0.1° C. and were recorded at two to three minute intervals from the first and fourth digits of each foot and from the right index finger. Rectal temperatures were taken by a special thermocouple and read to 0.01° C.

[1] *Manuscript received November 12, 1948.*

Contribution from the Departments of Surgery and Medical Research of the University of Manitoba and the Winnipeg General Hospital, Winnipeg, Man.

The subjects reclined in a constant temperature room (21° to 23° C.) and were clad in pyjamas. To provide a base line, recordings were made for 15 to 30 min. before a test. In approximately half of the cases Etamon was used prior to reflex vasodilatation and in the others the order was reversed. Between the tests sufficient time was allowed for skin cooling to occur and in some cases it was necessary to counteract the effects of reflex vasodilatation by immersing the left arm in cold water for a short time. As recommended by Coller *et al.* (3), Etamon was injected intravenously in doses of 300 and 500 mgm. in 40 to 60 sec. Within these limits advanced age, debility, and small stature were used as indications for the lower doses. Blood pressure and pulse rates were recorded at three minute intervals just prior to and for 15 min. following the injection.

Reflex vasodilatation was induced according to the method of Gibbon and Landis (5) by immersing the left arm in water at 44° C. and the heating effect was augmented by covering the trunk and proximal parts of the extremities with blankets. The subjects consisted of three normal young men and 11 patients with various types of vascular disease. These are detailed in Table I.

Results

The results of two experiments are illustrated in Figs. 1 and 2 to show the type of records used as a source for the data summarized in Table I. In

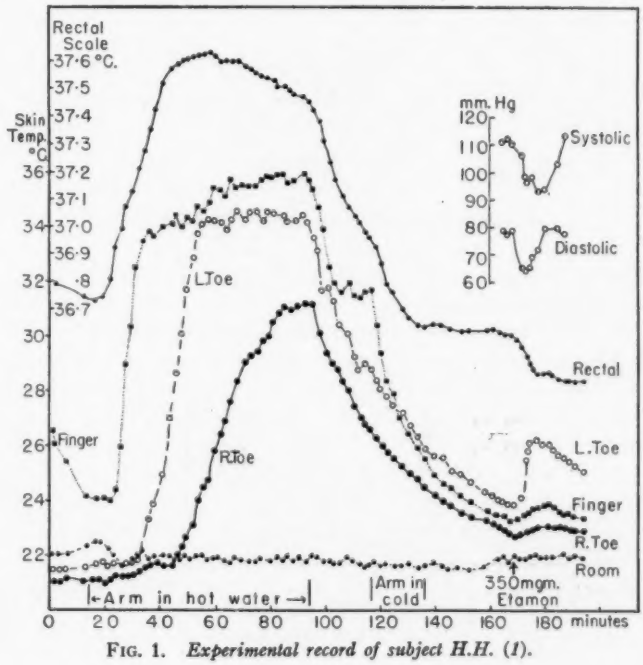

FIG. 1. *Experimental record of subject H.H. (1).*

TABLE I

EFFECT OF ETAMON CONTRASTED TO REFLEX VASODILATATION

Subject	Diagnosis	Sex	Age	Dosage		B.P., mm. Hg		Finger temp., °C.			Rt. toe temp., °C.			Lt. toe temp., °C.		
				Mgm.	Mgm./kgm.	Before Etamon	Fall	Reflex	Etamon	Diff.	Reflex	Etamon	Diff.	Reflex	Etamon	Diff.
R.L.	Livedo reticularis	F	19	425	8.8	105/ 62	20/ 2	36	33*	3	37	34*	3	37	35*	2
A.R. 1	Normal	M	22	400	4.4	130/ 70	20/ 10	37	24	13	35	21	14	35	21	14
A.R. 2	Normal	M	22	500	5.1	116/ 85	8/ 15	35	24*	11	35	32*	3	35	32*	3
B.H. 1	Normal	M	25	400	6.2	122/ 62	0/−14	35	32	3	35	29	6	35	28	7
B.H. 2	Normal	M	25	400	6.2	120/ 75	10/ 15	35	32	3	35	32	3	35	33	2
B.H. 3	Normal	M	25	400	6.2	120/ 70	10/ 8	35	35	0	34	34	1	35	34	1
D.P.	Hypertension	M	30	500	5.1	235/155	55/ 5	36	35	1	34	29	5	34	29	5
N.M.	Thromboangiitis	M	32	350	6.0	160/ 70	50/ 20	36	32	4	24	24	0	36	24	12
J.D.	Normal	M	38	400	5.5	105/ 80	10/ 5	36	29*	7	36	34*	2	36	34*	2
E.L.	Hypertension	F	55	350	5.3	230/130	115/ 45	36	25	11	33	31	2	33	26	7
H.H. 1	Thrombophlebitis	M	55	350	4.7	112/ 80	17/ 15	36	24	12	31	23	8	35	26	9
H.H. 2	Thrombophlebitis	M	55	400	5.5	122/ 80	24/ 14	35	27*	8	32	25	7	34	30*	4
J.McK.	Arteriosclerosis	M	57	300	5.2	140/ 75	48/ 15	36	23	13	24	22	2	24	22	2
A.N.	Thrombophlebitis	M	60	500	6.4	165/ 90	80/ 40	35	35	0	35	26	9	35	27	8
C.S. 1	Arteriosclerosis	M	63	400	5.3	150/ 85	32/ 20	36	31	5	23	21	2	34	33	1
C.S. 2	Arteriosclerosis	M	63	470	6.3	140/ 72	52/ 24	36	34	2	23	22	1	34	32	2
A.S.	Arteriosclerosis	M	63	355	5.0	112/ 75	47/ 65	37	22	15	30	20	10	34	21	13
G.H.	Arteriosclerosis	M	68	350	5.0	175/ 90	80/ 32	36	29*	7	34	31*	3	27	27	0
B.R.	Arteriosclerosis	M	77	350	4.7	128/ 80	33/ 20	36	34	2	35	28	7	35	24	11

* *Finger and toe effects of Etamon can be compared.*

this table the highest temperatures attained are stated and the subjects are listed in order of age. Omitted are the observations made on the fourth toes because they closely resembled those recorded for the great toes. It can be

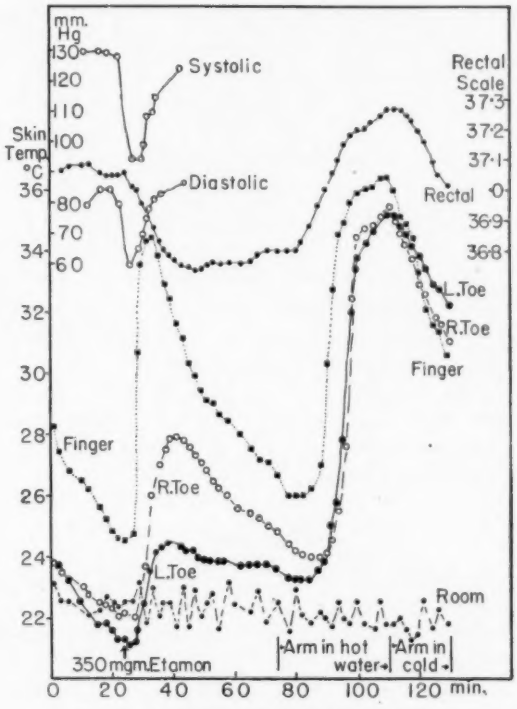

FIG. 2. *Experimental record of subject B.R.*

seen that in nearly every instance the level of skin temperature produced by reflex vasodilatation was greater than that caused by the intravenous injection of Etamon. The average maximum temperature of the finger with reflex vasodilatation was 6° C. greater than the average maximum temperature following Etamon. The corresponding difference in the right great toe was 5° C. and in the left great toe 6° C. Figs. 1 and 2 also show a fall in the rectal temperature following Etamon; the fall in temperature was present in all subjects with the exception of N.M. and averaged 0.2° C.

Table I shows that the discrepancy between the vasodilating action of heat and of Etamon differed greatly in the various subjects. This variation seemed to be due in part to the temperature of the digit when the Etamon was given and upon the size of the dose. To elucidate the influence of the initial temperature, three sets of observations with a constant dosage were made on subject B.H. In the first experiment reported on him in Table I, the initial

temperature of the toes was 21° C., and in the second it was 25° C., and in the third 29° C. It will be seen that with an increase in initial temperature a higher maximal temperature was obtained with a consequent decrease in the discrepancy between the effect of Etamon and that of reflex vasodilatation.

The effect of dosage can also be seen in Table I. Subjects A.R., H.H., and C.S., who each received two different doses showed a more marked elevation with the larger dose. However, when comparing one individual with another the degree of response did not bear such strict relationship to the size of the dose.

In regard to the difference in the response of the upper and lower limbs, all the data in Table I are not directly applicable because of the effects of initial temperature or the presence of arterial obstruction. In those subjects in whom the initial temperatures of the fingers and toes were similar and in whom there was no organic vascular disease (marked by asterisk in Table I), it will be seen that Etamon caused a greater effect upon the toes than the fingers. This confirms the observations of Larsson and Frisk (6).

It can be seen in Table I that Etamon frequently caused a striking fall in blood pressure in the older age group. Excluding the subjects with hypertension, those under fifty had an average fall in blood pressure of 16/8 mm. Hg while for those above fifty the average fall was 46/27 although the latter received in most instances a smaller dose.

Discussion

The observations reported here indicate that Etamon is not a satisfactory vasodilating agent because the temperatures achieved did not bear a consistent relationship to the dilatation potential demonstrated to be present by the method of reflex vasodilatation. It is true that Larsson and Frisk (6) found Etamon as satisfactory as lumbar block, but they used doses of 10 mgm./kgm., which are approximately 50% larger than those used by Boyd *et al.* (2) and by Coller *et al.* (3).

In the three cases in which Larsson and Frisk (6) compared the action of these large doses to that of body heating, no superiority of either method was demonstrated. However, as they state that doses of less than 10 mgm./kgm. do not produce maximal dilatation, it is apparent that the present results are more in agreement with theirs than with those reported by Coller *et al.* (3). The discrepancies are difficult to explain but may be related to a difference either in the initial temperature of the limbs or to a difference in room temperature. It should also be noted that the illustrations of Coller *et al.* (3) show the greatest effect in 20 or 30 min. whereas the present observations and those of Larsson and Frisk showed the peak of the temperature rise to be achieved in less than 10 min.

Moreover, in the absence of a total autonomic block it is highly probable that the fall in rectal temperature and the fall in the blood pressure would induce peripheral vasoconstriction and thus tend to counteract the peripheral

vasodilating action of Etamon. From this arises the necessity of using doses capable of producing complete blockade. Even 10 mgm./kgm. doses in the hands of Larsson and Frisk (6) did not produce results comparable to peripheral nerve blockade in four out of eight cases. The objection to doses of this size is the very marked fall in blood pressure, which is particularly pronounced in the arteriosclerotic group where vasodilation tests find their most important use. Because of the possibility that hypotensive interludes might produce serious complications in these subjects, it has not been considered advisable to explore the value of such large doses.

If such caution is warranted, it is apparent that the present results substantiate the opinion of DeBakey (4) and the findings of Boyd *et al.* (2) that Etamon is not a reliable agent for testing the vasodilatation potential of the peripheral circulation.

Acknowledgments

The equipment used in this study was furnished partly by the National Research Council of Canada and partly by the Manitoba Institute for the Advancement of Medical Education and Research. The Etamon was kindly supplied by Parke Davis Co. Mr. Arnold Rogers contributed valuable technical assistance.

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PLASMA MAGNESIUM IN DISORDERS OF THE THYROID GLAND[1]

By J. B. R. Cosgrove and W. F. Perry

Abstract

There is a conflict of evidence concerning the alterations of nondiffusible plasma magnesium in disorders of the thyroid gland. In this investigation it was found in 12 normal individuals, 9 patients with hyperthyroidism, 10 with myxedema, 6 with nontoxic goiter, and 14 patients with miscellaneous diseases, that the nondiffusible plasma magnesium was relatively constant in a wide range of conditions and was not altered even in patients with marked thyroid dysfunction.

Introduction

The total plasma magnesium can be separated into diffusible and nondiffusible fractions. While there is no reason to believe that the total value is altered in disorders of the thyroid gland, it has been claimed that the nondiffusible fraction is increased in hyperthyroidism and reduced in myxedema (4, 8, 9). However, normal values have also been reported in hyperthyroidism (2, 3). Because of the important role played by magnesium in many metabolic processes, this study was undertaken to determine whether or not alterations in the amount of nondiffusible magnesium occur in dysthyroid states.

Method

Two ml. of plasma, obtained by centrifugation of heparinized venous blood from fasting patients, was used for total magnesium determinations. To obtain the diffusible fraction 3 ml. was subjected to ultrafiltration. The value for the nondiffusible magnesium was obtained by subtracting the diffusible fraction from the total.

Magnesium was measured by the titan yellow method described by Kunkel *et al.* (6). The preparation of the standard solution was modified by preparing it with magnesium metal rather than magnesium sulphate septahydrate so as to avoid error due to the deliquescent properties of the salt. Nineteen mgm. of the metal was dissolved in 0.5 ml. of concentrated hydrochloric acid and diluted to 1 liter with distilled water. Owing to a change in the titan yellow solution it was found necessary to prepare a calibration curve daily. To determine the reliability of the method, eight magnesium determinations were made on the same plasma. A mean of 2.48 mgm. % was obtained with a range of 2.41 to 2.58 and a standard deviation of $\pm 0.05\%$. In 10 recovery experiments, 19 μgm. of magnesium was added to 2 ml. of plasma. The mean recovery of added magnesium was 100% with a range of 96 to 106%.

[1] *Manuscript received October 25, 1948.*

Contribution from the Departments of Medicine and Medical Research of the University of Manitoba and the Winnipeg General Hospital.

Utilizing an ultrafiltration apparatus of the type described by Nicholas (7), plasma was filtered through No. 300 wet cellophane using an air pressure of 125 lb. per sq. in. at a room temperature of 22° to 28° C. By placing the apparatus in an automatic shaker, 1 ml. of ultrafiltrate was obtained in approximately one hour. The performance of the filter was checked by testing all filtrates for protein with trichloroacetic acid. The reliability of the results was ascertained by showing that a second passage of an ultrafiltrate did not alter its concentration of magnesium, and in addition, 17 ultrafiltrates from the same sample of plasma had a mean magnesium value of 1.29 mgm. % with a range of only 1.18 to 1.38 and a standard deviation of ±0.05%. The concentration of magnesium in the ultrafiltrate was found not to be affected by varying the air pressure, soaking the cellophane in distilled water for varying periods, using oxalate instead of heparin as the anticoagulant, using serum instead of plasma, varying the amounts of plasma in the ultrafilter, using or not using the automatic shaker.

Five groups of subjects were studied. Those in the four abnormal groups were selected as having typical examples of their respective disorders. The diagnosis of the dysthyroid states was confirmed by basal metabolic readings and plasma protein-bound iodine determinations. The normal group consisted of six females and six males who were laboratory technicians or medical students with an age range of 20 to 30 years. The hyperthyroid group was made up of seven females and two males, ranging in age from 26 to 62, with a mean B.M.R. of +49% (range +29 to +78) and a mean protein-bound iodine value of 17.5 μgm. % (range 12.8 to 20.0). The nontoxic nodular thyroid group consisted of six females ranging in age from 30 to 79, with a mean B.M.R. of +3% (range −6 to +21) and a mean protein-bound iodine of 5.5 μgm.% (range 4.8 to 7.3). The hypothyroid group was composed of seven females and three males, varying in age from 18 to 72 years, with a mean B.M.R. of −35% (range −23 to −47) and a mean protein-bound iodine of 1.6 μgm. % (range 0.0 to 3.7 μgm.). The group of patients with miscellaneous diseases consisted of 10 females and 4 males with an age range of 6 to 71 years. The plasma protein-bound iodine was within the normal range (4 to 9 μgm. %). The B.M.R. was normal except in the cases of congestive heart failure and leukemia in which it was elevated. In the group were five patients with psychoneurosis and single examples of Paget's disease, cirrhosis of the liver, psychosis, infectious hepatitis, congestive heart failure, essential hypertension, carcinoma of the pancreas with jaundice, myelogenous leukemia, and diabetes mellitus.

Results

The levels of total plasma magnesium and its fractions in the various groups are shown in Table I. It is apparent that the nondiffusible fraction of the various groups showed no significant difference in either level or in

TABLE I

PLASMA MAGNESIUM IN VARIOUS GROUPS

Group	Number of cases	Mean total, mgm. % ± S.d. and range	Mean diffusible, mgm. % ± S.d. and range	Mean nondiffusible, mgm. % ± S.d. and range*	Mean % nondiffusible, ± S.d. and range**
Normal	12	2.60 ± 0.06 (2.50 to 2.71)	1.28 ± 0.12 (1.13 to 1.48)	1.30 ± 0.12 (1.11 to 1.50)	50.9 ± 4.6 (43.0 to 56.5)
Miscellaneous	14	2.66 ± 0.19 (2.33 to 2.98)	1.26 ± 0.12 (1.07 to 1.50)	1.13 ± 0.22 (1.01 to 1.80)	52.3 ± 5.7 (40.2 to 62.1)
Hyperthyroid	9	2.58 ± 0.19 (2.23 to 2.80)	1.32 ± 0.12 (1.14 to 1.55)	1.25 ± 0.19 (1.01 to 1.52)	48.3 ± 4.9 (41.8 to 57.1)
Nontoxic nodular thyroid	6	2.57 ± 0.14 (2.35 to 2.70)	1.24 ± 0.13 (1.08 to 1.44)	1.32 ± 0.11 (1.15 to 1.43)	51.4 ± 3.9 (46.4 to 55.7)
Hypothyroid	10	2.75 ± 0.19 (2.56 to 3.05)	1.32 ± 0.11 (1.20 to 1.54)	1.43 ± 0.12 (1.32 to 1.73)	52.1 ± 2.7 (47.4 to 56.6)

* *Difference between total and diffusible.*
** *Percentage of total.*

percentage relationship to the total. The values for the total plasma magnesium also were similar for the various groups, the highest being found in myxedema.

Discussion and Summary

In considering the above results it should be noted that the values for total plasma magnesium are slightly higher than those found by Kunkel *et al.* (6) who also used the titan yellow method. This may be due to the difference in standard solutions. However, the values reported here are well within the accepted range of 2 to 3 mgm. %. It is of interest that the values for total magnesium in the myxedematous group were slightly higher than in the normal group. This may bear some relationship to recent evidence that shows that a high total serum magnesium is associated with subnormal body temperature (10, 11, 12).

It is customary to express the value for nondiffusible magnesium as a percentage of the total. In the present investigation this was found consistently to be in the region of 50%. This value is at variance with previous results. Soffer *et al.* (8, 9) report for normals a mean of 14.5% (range 3.1 to 22.1), Cope and Wolff (3) found a mean of 37% (range 14 to 56), Dine and Lavietes (4) found a range of 14 to 31%, while Bissell (1) reports a mean of 30.4% (range 17 to 42). This difference in results must indicate some difference in technique or methods. The above authors used the method of Briggs (2) or some modification of it to determine the magnesium concentration. As this method necessitates the precipitation of calcium, there is a possibility that this precipitation may affect the subsequent determination of magnesium.

In the titan yellow method, calcium in the concentration found in human plasma does not affect the estimation of magnesium (5). This may be the explanation for the variance of results.

Whatever the correct explanation may be for the discrepancy between the present results and those of other investigators, the consistency of the results reported here gives confidence to the conclusion that neither the total nor the nondiffusible plasma magnesium is affected by disorders of the thyroid gland.

Acknowledgments

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AN EFFECT OF ILL HEALTH ON THE EXCRETION OF 17-KETOSTEROIDS[1]

By William F. Perry

Abstract

The urinary excretion of 17-ketosteroids was measured during the first four hours after waking and during the next 20 hr. of the day in 29 ill adults, 18 males and 11 females, and in 31 control adults, 16 males and 15 females. The ill subjects excreted a smaller fraction of their total daily output in the first four hours of the day than did the controls. This finding is ascribed to defective secretion of 17-ketosteroid precursors by the adrenal cortex.

Introduction

Various workers (6, 10, 14, 15) have demonstrated that the urinary excretion of 17-ketosteroids is usually lowered during sleep. Pincus (8, 9), besides noting decreased excretion during sleep, noted that ketosteroids were excreted at a higher rate during the morning hours than during the remainder of the day He has recently (10) also shown that this diurnal rhythm of excretion is only slightly affected by aging.

Several investigators (3, 4, 6, 13) have noted that ill health is associated with lowered excretion of urinary 17-ketosteroids. Most of these determinations have been carried out on 24-hr. samples of urine or on urine produced during shorter intervals but expressed on a 24-hr. basis. Therefore it was considered of interest to study the effect of ill health not only on the total 24-hr. production of ketosteroids but also on the reported diurnal rhythm in excretion and particularly the effect of ill health on the morning rise in ketosteroid excretion noted by Pincus (8, 9).

Methods

Because there is considerable daily variation in ketosteroid excretion (6, 14), urine was collected from most subjects on at least two separate days in a seven-day period. The mean figures for these days were used in calculating the results.

On waking, the subject discarded the overnight urine, and all urine produced during the next four hours was collected and assayed for its ketosteroid content. The urine produced during the next 20 hr. was collected separately and also assayed. Thus the total 24-hr. production could be obtained. Breakfast was taken early in the four-hour collection period; the other meals were taken during the 20-hr. collection period. The urine was either processed immediately after collection or stored in the frozen state. Urine stored in this manner has been found to undergo no deterioration in ketosteroid content for as long as four months.

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Contribution from the Departments of Medical Research of the University of Manitoba and the Winnipeg General Hospital, Winnipeg, Man.

Estimations of the 17-ketosteroid content were made using the hydrolysis and extraction procedure of Miller, Mickelsen, and Keys (7). Colorimetry was performed on the extracts using the modifications suggested by Cahen and Salter (2) to eliminate nonspecific chromogens. By this method, duplicate determinations checked within 5%. Androsterone* was used as a reference standard. Standard statistical methods as described by Fisher (5) and Snedecor (12) were used in analyzing the results.

Subjects

All the hospitalized subjects were patients on the wards of the Winnipeg General Hospital.

A. Controls

(i) Sixteen males of whom eight, ranging in age from 20 to 23 years, were normal people at their usual occupations; the other eight, ranging in age from 35 to 64 years, were hospitalized for various elective operations or investigational procedures, but their general health was not affected. These subjects are listed in Table I.

(ii) Fifteen females aged 16 to 61 years; three of these were working at their usual occupations; the other 12 were hospitalized for investigational procedures similar to those for some of the control males and, like the control males, they did not appear to be ill in a general sense. Their illnesses consisted of two cases each of bunions, minor injuries, psychoneurosis, and nontoxic goiter; one case each of pregnancy, fractured pelvis (one month after injury), appendicitis (one week postoperative), and quiescent rheumatoid arthritis.

In both control groups ketosteroid determinations were done either prior to any operative procedures or at a sufficient time afterward to avoid the elevation of ketosteroid excretion that follows operative procedures and other stress situations (6).

B. *Ill Subjects*

(i) Eighteen males aged 24 to 85 years. These patients and the diagnoses of their illnesses are listed in Table II.

(ii) Eleven females aged 16 to 70 years. In this group the illnesses were: two cases each of myxedema and Grave's disease and one case each of subacute appendicitis, pulmonary edema, hemiplegia, carcinoma of the pancreas, carcinoma of the stomach, carcinoma of the rectum, and Cushing's syndrome.

The subjects in these two groups were designated ill on the basis of clinical criteria. In general they had lowered hemoglobin levels and elevated sedimentation rates in contrast to the control group in which these values were

** The Androsterone used in this investigation was kindly donated by the CIBA CO., LTD., Montreal, Que.*

usually normal. The ill subjects showed no evidence of renal failure as judged from blood urea nitrogen values and from the variability in urine concentration.

Results

The individual and average 4-, 20-, and 24-hr. excretions of 17-ketosteroids by the 16 control males are shown in Table I. The four-hour excretion is also shown as a percentage of the total 24-hr. excretion. The mean value of these percentages for the control group of 16 males was 22.5%.

TABLE I

EXCRETION OF 17-KETOSTEROIDS BY 16 CONTROL MALES*

Name	Age	Diagnosis	4-Hr. excretion, mgm.			Mean 4-hr. excretion	20-Hr. excretion, mgm.			Mean 20-hr. excretion	Mean 24-hr. excretion	Mean 4-hr. as % 24-hr. excretion
			1	2	3		1	2	3			
G.B.	20	Normal	2.1	3.0	2.5	2.5	11.0	10.7	11.0	10.9	13.4	18.7
M.B.	26	Normal	3.4	3.5	2.1	3.0	11.8	12.4	11.2	11.8	14.8	20.2
R.C.	27	Normal	3.3	4.0	3.1	3.4	11.3	17.9	14.2	14.4	17.8	19.0
J.C.	24	Normal	3.0	3.2	2.4	2.9	11.1	10.4	11.4	10.9	13.8	21.0
W.P.	28	Normal	2.3	3.7	3.8	3.2	9.6	9.8	10.5	9.9	13.2	24.2
C.K.	33	Normal	3.3	4.3	2.9	3.5	7.1	7.8	14.3	9.7	13.2	26.5
R.D.	24	Normal	3.1	3.2	5.2	3.5	12.5	9.0	9.9	10.8	14.3	24.5
L.K.	20	Normal	4.4	4.8	–	4.6	11.1	15.5	—	13.3	17.9	25.6
H.C.	36	Malunited fracture	1.3	–	–	1.3	4.3	—	—	4.3	5.6	23.2
P.L.	53	Auricular fibrillation	2.0	–	–	2.0	7.2	—	—	7.2	9.2	21.7
H.M.	41	Intervertebral disc	2.3	–	–	2.3	7.5	—	—	7.5	9.8	23.5
N.P.	37	Fractured tibia (one month later)	2.8	1.9	1.9	2.2	8.6	9.3	6.2	8.0	10.3	21.4
C.S.	62	Medial meniscus	2.6	2.2	–	2.4	4.7	5.8	—	5.2	7.6	31.5
T.S.	35	Intervertebral disc	2.5	–	–	2.5	10.2	—	—	10.2	12.7	19.7
H.W.	64	Bronchial asthma	1.4	1.2	0.7	1.1	4.9	2.4	3.9	3.7	4.8	22.9
N.G.	31	Nontoxic goiter	1.9	2.0	–	1.9	4.7	10.4	—	7.6	9.5	20.0
Mean values						2.2				9.1	11.7	22.5

In Table II are presented the individual and average 4-, 20-, and 24-hr. excretions of the 18 sick males, each four-hour excretion being again shown as a percentage of the total 24-hr. excretion. In four hours these 18 ill males excreted on the average only 14.7% of their 24-hr. output.

In Table III is presented a summary of the data obtained on these two groups and of analogous data obtained on the two groups of females. It will be seen that the control females had an average four-hour excretion of 19.3% of their total 24-hr. output, while the sick females averaged only 14.5%.

The ill subjects excreted smaller absolute amounts of ketosteroids than the well. This is in agreement with previous findings (3, 4, 6, 13). Because there was considerable variation in the fraction excreted in the first four hours

TABLE II

EXCRETION OF 17-KETOSTEROIDS BY 18 ILL MALES

Name	Age	Diagnosis	4-Hr. excretion, mgm.			Mean 4-hr. excretion	20-Hr. excretion, mgm.			Mean 20-hr. excretion	Mean 24-hr. excretion	Mean 4-hr. as % 24-hr. excretion
			1	2	3		1	2	3			
I.I.	71	Prostatic hypertrophy	0.45	0.58	0.56	0.53	4.6	3.7	4.2	4.3	4.8	11.0
W.J.	61	Prostatic carcinoma	0.54	0.55	—	0.55	4.2	5.7	–	4.4	4.9	11.2
W.L.	67	Prostatic carcinoma	1.10	1.40	0.63	0.95	5.4	4.8	4.4	5.1	6.0	15.8
H.McI.	69	Prostatic hypertrophy	2.60	—	—	2.60	5.5	–	–	5.5	8.1	32.0
W.McN.	50	Prostatic carcinoma	1.10	1.40	—	1.30	6.4	6.8	–	6.4	8.0	16.2
W.F.	35	Pelvic fracture	0.47	1.80	0.39	0.88	5.5	2.6	5.6	4.9	5.8	15.2
R.McR.	77	Prostatic hypertrophy	1.00	0.70	1.00	0.90	7.9	7.6	5.7	7.0	7.9	11.4
L.S.	64	Cirrhosis	0.34	—	—	0.34	5.1	–	–	5.1	5.4	6.4
P.M.	35	Addison's disease	0.62	—	—	0.62	4.0	–	–	4.0	4.6	13.4
R.B.	24	Hepatitis	0.88	—	—	0.88	6.0	–	–	6.0	6.9	12.8
W.H.	71	Prostatic hypertrophy	0.95	—	—	0.95	7.0	–	–	7.0	7.9	12.0
J.P.	85	Prostatic carcinoma	0.50	0.60	0.60	0.56	3.0	3.9	3.7	3.5	4.1	13.7
H.W.	72	Prostatic hypertrophy	0.84	1.20	0.21	0.75	3.5	1.7	1.8	2.3	3.0	25.0
J.W.	74	Prostatic carcinoma	0.27	0.96	—	0.61	3.7	2.9	–	3.3	4.0	15.2
S.L.	73	Myxedema	0.32	0.57	0.36	0.32	3.4	4.7	3.0	3.7	4.1	7.8
S.S.	75	Prostatic hypertrophy	0.56	1.20	0.77 1.20	0.93	4.7	6.7	5.6 5.2	5.5	6.5	14.3
H.T.	72	Prostatic hypertrophy	0.74	0.79	0.82	0.79	5.3	6.9	5.2	5.8	6.6	12.0
H.B.	66	Malnutrition	0.40	—	—	0.40	4.1	–	–	4.1	4.5	8.9
Mean values						0.83				4.9	5.7	14.7

TABLE III

EXCRETION OF KETOSTEROIDS BY CONTROL AND ILL SUBJECTS

Group	Mean 4-hr. excretion, mgm.	Mean 20-hr. excretion, mgm.	Mean 24-hr. excretion	Mean 4-hr. excretion % of 24-hr. excretion	Standard deviation	Range
Control males (16)	2.20	9.1	11.7	22.5	3.3	18.7 – 31.5
Sick males (18)	0.83	4.9	5.7	14.7	6.0	7.8 – 32.0
Control females (15)	1.39	5.8	7.2	19.3	4.0	11.4 – 25.7
Sick females (11)	0.83	5.2	6.0	14.5	3.4	10.2 – 21.6

in all groups, an analysis of variance was performed to test the significance of the difference in four-hour percentage excretions of the four groups. In Table IV are set forth the calculated probabilities of the differences in the four-hour percentage excretion being due to chance.

Thus there is a highly significant difference between the four-hour percentage excretions of the sick males (14.7%) and of the control males (22.5%) and also between the sick females (14.5%) and the control females (19.3%).

TABLE IV

SIGNIFICANCE OF DIFFERENCE BETWEEN GROUPS

Group	*P*
Control males vs. sick males	<.01
Control females vs. sick females	<.01
Control females vs. control males	>.01 but <.05
Sick males vs. sick females	>.05+

It would appear in addition that control males excrete a significantly higher fraction of their total 24-hr. production of ketosteroids in the first four hours of the day than do control females.

Discussion

Before discussing the interpretation of the results it is necessary to consider factors involved other than illness. The most apparent of these is hospitalization. That this does not explain the difference between the groups is shown in Table I. It will be seen that the normal active individuals had the same four-hour percentage excretion as the hospitalized subjects. This suggests that hospitalization per se does not substantially alter the fraction of the total ketosteroid output excreted in the first four hours. Defective kidney function could not be held to be a responsible factor.

The results reported above show that ill health, in addition to its generally accepted role in decreasing the absolute amounts of ketosteroids excreted, also tends to abolish the morning increase in excretion that is characteristic of the healthy state. The normal morning rise in excretion has been interpreted by Pincus (8, 9) as a reaction to the stress of daily activities that begin on waking. Forbes *et al.* (6) have shown that chronically ill hospital patients, unlike normal persons, are unable to respond to various stress situations by excreting greater amounts of ketosteroids. They ascribe this phenomenon in the healthy to a release of cortical "N" hormone from the adrenal cortex. The chronically ill do not respond in this manner and are considered to be in the resistant phase of the "Adaptation Syndrome" of Selye (11).

The altered diurnal rhythm of ketosteroid excretion in the group of ill subjects may therefore be due to lack of adrenal cortical response to the stress of the numerous, diverse activities that begin on waking. If so, the estimation of this fraction may be of some value in assessing the functional state of the androgenic or "N" part of the adrenal cortical system.

The moderately significant difference in the four-hour fractions of the control males and females suggests possibly a greater reactivity on the part of the male. However, this difference does not appear large and its cause has not been investigated. Possibly, ketosteroids of testicular origin are also subject to variations in excretion as are those of cortical origin.

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PRESERVATION OF ACETYLCHOLINE IN HUMAN CEREBROSPINAL FLUID: A SIMPLE METHOD FOR CLINICAL USE[1]

By Donald B. Tower

Abstract

A simple and effective method for preservation of acetylcholine in human cerebrospinal fluid has been described. The method depends on the use of acidification and prostigminization of cerebrospinal fluid. No detectable loss of acetylcholine for periods up to seven days or longer has been observed. The method has been satisfactorily used in clinical investigations on more than 200 cerebrospinal fluid samples from over 100 neurological and neurosurgical patients.

Introduction

One of the principal difficulties in the study of the acetylcholine–cholinesterase system is the instability of the acetylcholine molecule. A satisfactory method for its preservation until assays are carried out is essential in biological work. Babkin *et al.* (1) have reported a method for the preservation of acetylcholine in whole blood for periods up to seven days. The method depends on the inhibition of cholinesterase activity by the combined use of prostigmine, acidification, and refrigeration. Considerable variability in results was encountered because of the difficulty of pH control in the presence of the large electrolyte and protein buffer of the blood and because of the large quantity of cholinesterases free to act before preservation could be achieved (3).

It has been possible to work out a successful modification of this method for cerebrospinal fluid because of three fundamental differences between cerebrospinal fluid and blood. *First*, the buffering power of cerebrospinal fluid is very small, so that acidification and maintenance of an acid pH present no problem. *Second*, the cholinesterase activity of cerebrospinal fluid is only about 0.5% of that of whole blood (5). And *third*, the observed substrate concentrations of 0.02 to 10 μgm.%* acetylcholine have proved to be suboptimal, so that enzyme activity is markedly impaired (5). Thus, the loss of acetylcholine before preservation is negligible.

Procedure

The method of acetylcholine preservation was developed for the assay procedure employing the isolated ventricle of the mollusc, *Venus mercenaria* (4), but it may be adapted to any of the other standard methods of assay.

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Contribution from the Department of Neurology and Neurosurgery, McGill University, and the Montreal Neurological Institute, Montreal, Canada.

This study was supported by a grant from the Rockefeller Foundation.

* *μgm.% = micrograms per 100 ml.*

(1) Freshly drawn cerebrospinal fluid (5.0 ml.) is pipetted into a clean test tube, containing 1.0 ml. of 1 : 4400 prostigmine (neostigmine) methyl sulphate, 1.0 ml. of 0.1 *N* hydrochloric acid, and 2.0 ml. of 0.9% sodium chloride. (The pH of the preserved solutions varies between 5.5 and 6.0, as measured with a Beckman pH meter.)

(2) After the contents are thoroughly mixed, the solution is refrigerated at about 5° C. until ready for use.

(3) Immediately prior to testing the solution, 1.0 ml. of 0.05 *N* sodium hydroxide is added to partially neutralize the acid (raising the pH to 6.9–7.3). Final volume of the preserved solution is 10.0 ml., which represents a 1 : 2 dilution of the original cerebrospinal fluid sample.

Cerebrospinal fluid samples preserved in this manner show no detectable loss of acetylcholine for periods of seven days or longer. The preservation formula is summarized in Table I.

TABLE I

FORMULA FOR PRESERVATION OF ACETYLCHOLINE IN HUMAN CEREBROSPINAL FLUID

Cerebrospinal fluid	5.0 ml.
Prostigmine, 1 : 4400	1.0 "
Hydrochloric acid, 0.1 *N*	1.0 "
Sodium chloride*, 0.9%	2.0 "
	9.0 ml. (pH = 5.5 to 6.0)
Sodium hydroxide, 0.05 *N* (to neutralize)	1.0 ml.
Final volume	10.0 ml. (pH = 6.9 to 7.3)

* *Optional.*

The 10.0 ml. volume was found most convenient to provide sufficient quantity for multiple tests of active and hydrolyzed samples.*

Results

More than 200 samples of human cerebrospinal fluid have been satisfactorily preserved by the method outlined above. As an example of its efficacy over a seven day period, one experimental run (PR-8) is illustrated. The make-up of the samples is tabulated in Table II.

* *The 2.0 ml. of 0.9% sodium chloride in the formula may be omitted, since it has been included only to give a 1 : 2 dilution. In calculating the concentrations of acetylcholine in the original cerebrospinal fluid sample, the volume of the assay chamber, the preservation dilution factor, and the amount of acetylcholine measured by assay are multiplied together. Thus, in the results reported here, where a 20.0 ml. assay chamber was used, the original cerebrospinal fluid samples contained 2 × 20 or 40 times the amounts of acetylcholine recorded at assay.*

TABLE II

EXPERIMENTAL PRESERVATION OF ACETYLCHOLINE IN HUMAN CEREBROSPINAL FLUID

(EXPT. No. PR-8)

MAKE-UP OF SAMPLES

Constituents	Concentration	Samples (vol. in ml.)						
		M-1	M-2	N-1	N-2	O	P-1	P-2
Prostigmine	1:4400	1.0	1.0	—	—	1.0	1.0	1.0
Hydrochloric acid	0.1*N*	1.0	1.0	—	—	1.0	1.0	1.0
Sodium chloride	0.9%	—	—	3.0	3.0	2.0	5.0	5.0
Pooled human CSF	—	5.0	5.0	5.0	5.0	5.0	—	—
Acetylcholine	10.0 μgm.%	2.0	—	2.0	—	—	2.0	—
Acetylcholine	5.0 μgm.%	—	2.0	—	2.0	—	—	2.0
Sodium hydroxide*	0.05*N*	1.0	1.0	—	—	—	—	—
Sodium hydroxide*	0.1*N*	—	—	—	—	1.0	1.0	1.0
Total volume		10.0	10.0	10.0	10.0	10.0	10.0	10.0
Calculated final concentration of acetylcholine in μgm.% (for 1.0 ml. sample in 20.0 ml. chamber)		0.1	0.05	0.1	0.05	0.0	0.1	0.05

* *Added immediately before testing.*

A pool of four human cerebrospinal fluids, previously shown to contain no acetylcholine, was used. A series of identical samples for testing on successive days was set up as indicated (Table II).

The results of assay after 24 hr. of refrigeration are shown in Fig. 2. It can be seen that unpreserved samples, N-1 and N-2, showed little or no acetylcholine remaining after 24 hr. They gave a *Venus* heart tracing similar to that of Sample O, which contained no acetylcholine. In contrast, Samples M-1, M-2, P-1, and P-2 produced the same percentage of inhibition of the *Venus* heart beat as fresh aqueous known solutions of the same concentration. This is graphically demonstrated in Fig. 1, where the measured percentage inhibition of the *Venus* heart beat (in Fig. 2) has been plotted against the acetylcholine concentration in μgm.%. The correspondence of the points for fresh aqueous known solutions and for preserved M and P solutions of the same concentration shows no detectable loss of acetylcholine to have occurred in 24 hr.

In Fig. 3 a portion of the *Venus* heart tracing after seven days, preservation shows that in preserved samples M-1 and M-2 no detectable loss of acetylcholine occurred, while in unpreserved samples N-1 and N-2 no detectable acetylcholine remained. Several similar experiments were run, all of which showed satisfactory preservation of known amounts of acetylcholine in human cerebrospinal fluids for periods up to 10 days.

In practice unknown cerebrospinal fluid samples from ward patients have all been tested within seven days of the time of withdrawal and preservation of the sample. *Venus* heart tracings on two such cases of epilepsy with posi-

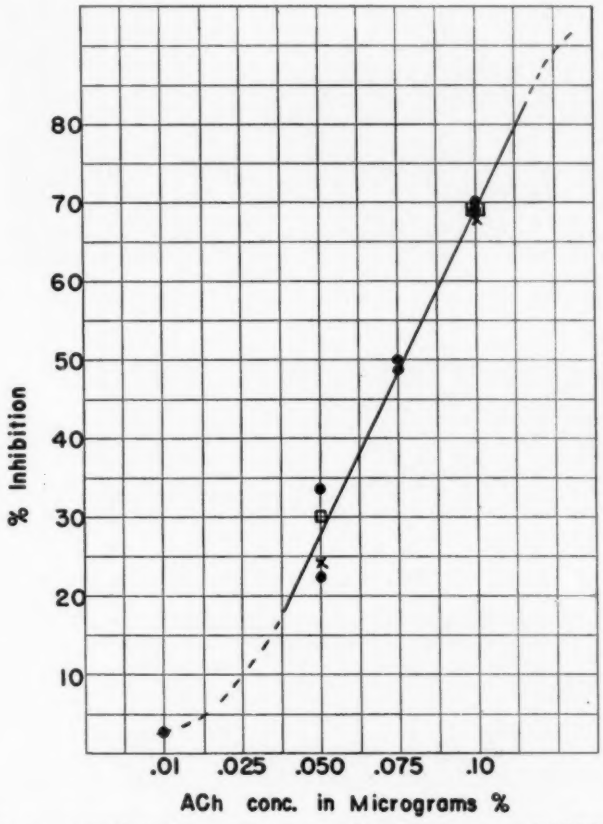

FIG. 1. *Acetylcholine concentrations plotted against percentage inhibition of assay test object for known fresh aqueous solutions and preserved solutions, derived from Fig. 2. M-5161.* ● = *Fresh solutions.* □ = *P (preserved solutions).* × = *M (preserved solutions).*

tive cerebrospinal fluid acetylcholine assays are given in Fig. 4. The results of cerebrospinal fluid studies on 130 neurological and neurosurgical patients and on experimental animals, using this method of acetylcholine preservation, will be reported elsewhere (2, 5, 6, 7).

Acknowledgments

The author wishes to express his appreciation for the advice of Dr. Donald McEachern and the assistance of Mr. Murray Bornstein in carrying out these experiments.

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Explanation of Figures

PLATE I

FIG. 2. *Acetylcholine assays on human cerebrospinal fluid samples preserved for 24 hr. (Expt. PR-8).*

ACh = Acetylcholine in μgm.%
w = Wash
M, N, O, P = Preserved and unpreserved samples (See Table II)
h = Hydrolyzed samples.

PLATE II

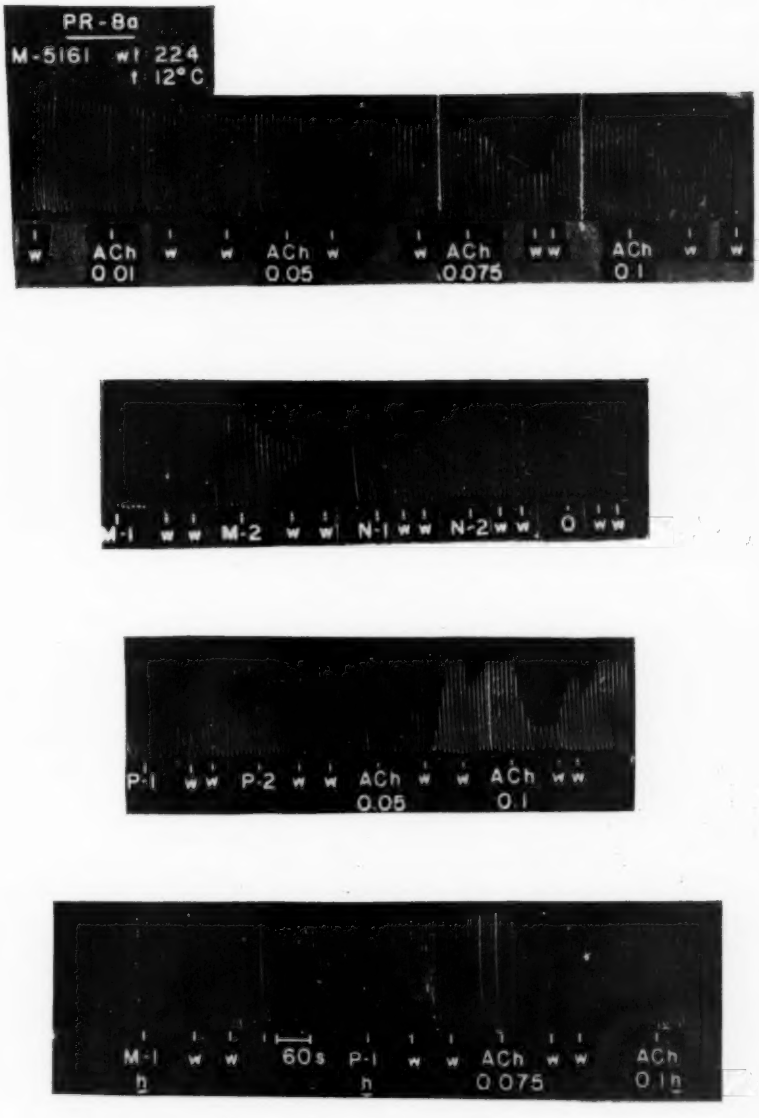
FIG. 3. *Acetylcholine assays on human cerebrospinal fluid samples preserved for seven days (Expt. PR-8).*

ACh = Acetylcholine in μgm.%
w = Wash
M, N, O = Preserved and unpreserved solutions (See Table II)
h = Hydrolyzed samples.

FIG. 4. *Acetylcholine assays on cerebrospinal fluids of human epileptic patients; samples preserved by the author's method.*

ACh = Acetylcholine in μgm.% (Note: 10^{-10} = 0.01 μgm.%)
w = Wash
h or hyd = Hydrolyzed samples
1217GN = Patient with focal left temporal lobe seizures, cause unknown
916RMacD = Patient with focal right temporal lobe seizures due to calcified ependymoma

PLATE I


PR-8a
M-5161 wt 224
t 12°C
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M-1 w w M-2 w w N-1 w w N-2 w w O w w
P-1 w w P-2 w w ACh 0.05 w w ACh 0.1 w w
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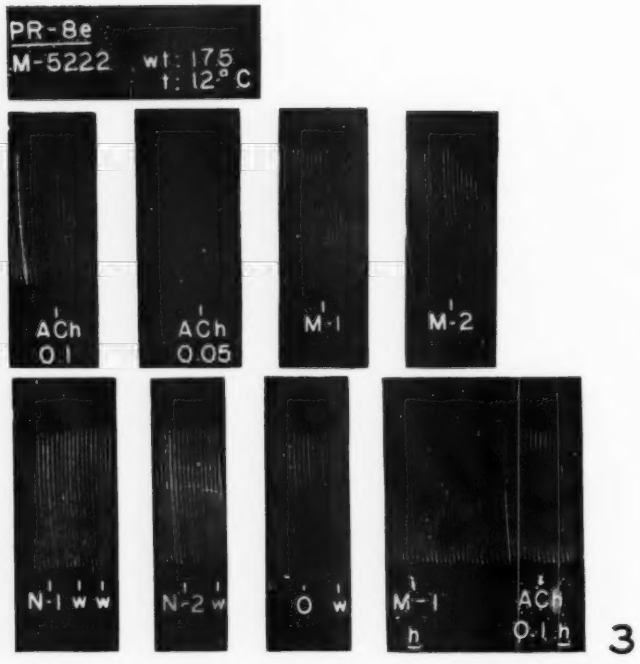
PR-8e
M-5222 wt: 175
t: 12.° C
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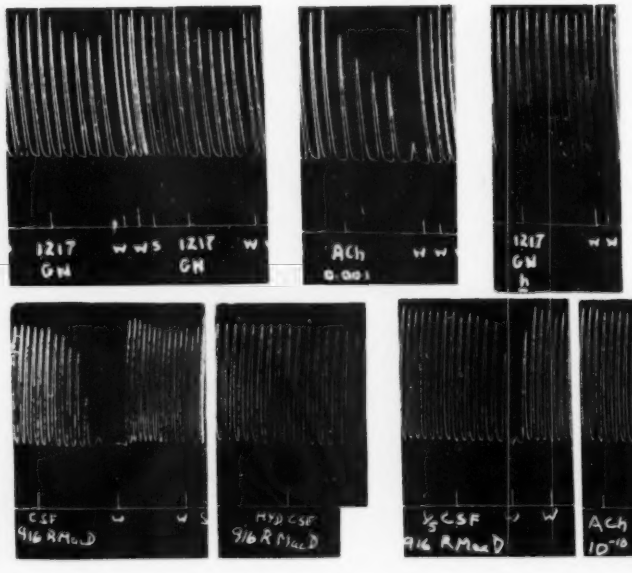
ACh 0.05
M-1
M-2
N-1 w w
N-2 w
O w
M-1 h
ACh 0.1 h

3


1217 GH
w w s
1217 GH
ACh 0.001
w w
1217 GH h
w w
CSF 9/6 RMgCl D
w w s
MYD CSF 9/6 RMgCl D
½ CSF 9/6 RMgCl D
w w
ACh 10⁻⁶
w w

4

THE RELATIONSHIP BETWEEN SERUM ALKALINE PHOSPHATASE AND DIETARY ESSENTIAL AMINO ACIDS[1]

By Jules Tuba, Max M. Cantor, and A. Gerald Richards

Abstract

It has been found that, when weanling rats are placed on a diet containing low protein barley (protein = 8.5%) or on diets containing low protein barley with supplements of casein or individual essential amino acids, with the exception of lysine and methionine, the level of serum alkaline phosphatase is related in an inverse fashion to the rate of growth. The addition to the basal low protein diet of methionine or casein, i.e., of labile methyl groups, results in the lowering of serum alkaline phosphatase activity towards normal levels. This is considered to reflect a return to normal of fat mobilization.

Introduction

It was reported by Hough *et al.* (4, 5) that dogs fed a high fat, protein deficient diet showed a pronounced elevation of serum alkaline phosphatase values and that these levels decreased following the return of the animals to a diet containing adequate protein. They presented evidence that the lipotropic substances, choline and methionine, reverse the effect of protein deficiency on serum phosphatase. It is apparent from their work that fat metabolism is related to serum phosphatase activity and that the phosphatase-decreasing action of proteins is associated with the methionine content of the diet. A direct correlation between the activity of serum alkaline phosphatase and the amount of fat ingested has been shown clearly by Cantor *et al.* (1). They showed that a decrease below full daily requirements in the food consumption of adult male rats produced a fall in serum alkaline phosphatase proportional to the amount of fat consumed daily rather than to the lowered calorie intake.

The work reported here is a preliminary study designed to determine the effect on serum alkaline phosphatase levels in growing rats of: (1) a low protein diet, (2) a low protein diet supplemented with each of eight essential amino acids.

Experimental

Weanling male albino rats, 21 days old, were used for the feeding experiments. They were housed individually in metal cages and given tap water to drink. They were fed daily at 9 a.m. except on days when blood samples were taken and then food was provided immediately after bleeding. There were at least six animals in each of the groups used for the various diets.

[1] *Manuscript received August 9, 1948.*

Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta., with financial assistance from the National Research Council, Ottawa, Canada.

Tail blood was collected by "milking" about 0.5 ml. of blood into micro-centrifuge tubes for obtaining serum. Within an hour of collection, serum was removed and stored in a refrigerator. Enzyme activity was invariably estimated within two or three days. Serum phosphorus and alkaline phosphatase* were determined by the Gould and Schwachman modification (3) of the Shinowara micro-method (8). Weights of individual animals in each group were recorded twice a week during the feeding period, which was generally about six weeks, and gain or loss in weight was noted. These changes were averaged for each group.

The basal diet was finely ground low protein barley (L.P.B.) containing 9.1% protein and 3% fat, supplemented with fat, salt mixture, and vitamins in the following proportions:

L.P.B.	930 gm.
McCollum's salt mixture	40 gm.
Crisco	30 gm.
Cod liver oil	20 gm.

To this was added 10 mgm. thiamine hydrochloride, 10 mgm. pyridoxine hydrochloride, 20 mgm. calcium pantothenate, 10 mgm. niacin, and 1 gm. choline chloride, all dissolved in 15 ml. of 95% alcohol; and 20 mgm. riboflavin distributed in 5 gm. of glucose. All the ingredients were mixed intimately and passed through a fine wire sieve three times. This basal diet, containing 8.5% protein, was stored in tightly stoppered dark bottles.

For the supplemented diets the following amounts of individual amino acids and of casein were contained in 1 kgm. of each diet:

Lysine monohydrochloride	7.80 gm.
Leucine	2.30 gm.
dl-Methionine	8.80 gm.
dl-Phenylalanine	5.20 gm.
dl-Threonine	5.20 gm.
Tryptophane	1.86 gm.
Valine	1.86 gm.
Histidine monohydrochloride	3.00 gm.
Casein (SMACO vitamin-free)	80.00 gm.

The barley and an estimate of its essential amino acid content were kindly supplied to us by the Department of Animal Science, University of Alberta. Each amino acid was added to the basal diet in an amount sufficient to raise the level of the acid present in L.P.B. and supplement to the requirement set

** The unit of phosphatase activity is equivalent to 1 mgm. of phosphorus liberated as phosphate ion during one hour of incubation at 37° C. with a substrate containing sodium β-glycerophosphate at pH for the alkaline enzyme of 9.3.*

by Rose (6) on the basis that food consumption was 10 gm. per animal per day. The addition of 8% casein to the basal diet raised the protein content to 16.5% and increased the daily intake of essential amino acids to approximately that required by Rose. Where the racemic form of the amino acid was used, the calculated amount was doubled. Arginine was not added to the basal diet since the barley contained a large excess of this acid. A group of animals fed the stock laboratory diet of Purina Fox Checkers containing not less than 20% protein served as a control. All diets were fed for a period of 40 days.

The Effect of L.P.B. and Supplemented L.P.B. on the Ratf of Growth of Normal Weanling Rats

Data on these experiments is presented in Table I in terms of average weight gained per group at 10 day intervals.

TABLE I

The relationship between diet and weight gain

Diets	No. of animals	Weight gain in grams			
		Days on diet			
		10	20	30	40
Checkers	10	28	78	125	165
L.P.B.	20	10	22	36	53
L.P.B. and casein	10	31	75	102	120
L.P.B. and lysine	10	12	26	42	57
L.P.B. and leucine	6	12	29	40	46
L.P.B. and methionine	6	14	25	36	50
L.P.B. and threonine	6	10	22	35	48
L.P.B. and valine	6	15	32	47	62
L.P.B. and histidine	6	12	27	42	62
L.P.B. and phenylalanine	6	7	16	27	40
L.P.B. and tryptophane	6	6	15	26	40

Growth of weanling rats on L.P.B. is greatly retarded. The addition of casein to L.P.B. provides partial protein replacement but the supplementation of L.P.B. with individual amino acids produces no marked improvement in growth and, in some instances, notably with phenylalanine and tryptophane, there seems to be further retardation of growth.

The Effect of L.P.B. and Supplements on Serum Alkaline Phosphatase Activity in Normal Rats

Serum alkaline phosphatase was estimated at the commencement of feeding and at 3.0, 4.5, and 6 weeks. The results presented in Table II are for the same animals as those referred to in Table I and are the averages of groups.

TABLE II

SERUM ALKALINE PHOSPHATASE LEVELS IN SHINOWARA UNITS OF GROWING RATS ON LOW PROTEIN DIET AND SUPPLEMENTED LOW PROTEIN DIETS

Diets	Serum alkaline phosphatase units/100 ml.		
	3 Weeks	4.5 Weeks	6 Weeks*
Checkers (20% protein approx.)	108	132	104 ± 28
L.P.B. (8.5% protein)	186	187	179 ± 38
L.P.B. and casein (16.5% protein)	183	148	160 ± 25
L.P.B. and methionine	196	138	139 ± 13
L.P.B. and lysine	241	253	234 ± 49
L.P.B. and phenylalanine	192	214	212 ± 25
L.P.B. and tryptophane	256	243	203 ± 33
L.P.B. and histidine	251	215	191 ± 18
L.P.B. and leucine	196	220	169 ± 16
L.P.B. and threonine	201	168	164 ± 26
L.P.B. and valine	237	235	178 ± 31

* *Standard deviations are indicated for phosphatase values at end of experiment.*

It will be seen that at the termination of the experiment animals fed the L.P.B. diet showed an elevation of about 75% in serum alkaline phosphatase activity as compared with the normal animals given the stock laboratory diet. Supplementing the L.P.B. with casein reduced alkaline phosphatase activity slightly below the values for the L.P.B. group. There is a marked decrease in enzyme activity with methionine. A further increase in phosphatase activity above that noted in the L.P.B. group is present in animals receiving supplements of lysine, phenylalanine, and tryptophane. Animals receiving the other supplements showed no marked change from those in the L.P.B. group.

EFFECT OF CHANGING ANIMALS FROM L.P.B. PLUS LYSINE DIET TO STOCK DIET

Table III presents the changes in phosphatase activity and rate of weight gain in 10 animals. These animals were kept on the L.P.B. and lysine diet for 40 days and then returned to the stock laboratory diet for eight days.

TABLE III

EFFECT OF NORMAL DIET ON ANIMALS PREVIOUSLY FED L.P.B. AND LYSINE

	L.P.B. + lysine diet			Stock diet
	21 Days	30 Days	40 Days	48 Days
Alkaline phosphatase units per 100 ml.	241	253	234	131
Weight gain in grams	12	26	57	98

It appears from Table III that the increase in alkaline phosphatase is related to the degree of retardation in growth. After nearly six weeks on L.P.B. and lysine, phosphatase activity was 234 units per 100 ml. of serum while the weight gain was only 57 gm. In the eight day period following the feeding of the stock laboratory diet, the phosphatase activity was reduced to an almost normal level and there was a weight gain of 41 gm.

Table IV presents a compilation of the changes in phosphatase activity and growth at the end of 40 days of the test period on the various diets used.

TABLE IV

THE RELATIONSHIP BETWEEN GROWTH RATE ON VARIOUS DIETS AND SERUM ALKALINE PHOSPHATASE ACTIVITY

Diet	Weight gain in 40 days	Alkaline phosphatase units/100 ml.
Stock diet	165	104
L.P.B. and casein	120	160
L.P.B. and valine	69	178
L.P.B. and histidine	62	191
L.P.B. and lysine	57	234
L.P.B.	53	180
L.P.B. and methionine	50	139
L.P.B. and threonine	48	164
L.P.B. and leucine	46	169
L.P.B. and phenylalanine	40	212
L.P.B. and tryptophane	40	203

It will be seen that groups fed L.P.B. and phenylalanine or L.P.B. and tryptophane showed the least weight gain and the highest phosphatase levels apart from the group on L.P.B. and lysine. In general, animals in other groups showed a similar correlation, and the less the weight gain, the higher the phosphatase level with the exception of the groups maintained on L.P.B. and methionine or lysine supplements.

The behavior of lysine in departing from the general trend cannot be explained. It should be noted, however, that this amino acid has been shown to be important for the synthesis of proteins in the liver by Gillespie *et al.* (2), and it is possible that alkaline phosphatase functions in such processes.

The effect of methionine used to supplement a diet low in protein is very definite and this observation is in conformity with the findings of Hough and his co-workers. Hough fed to dogs a diet containing 33% fat, a level much higher than 8% fat received by the rats used in the work reported here. Nevertheless, it seems that in both instances the increased phosphatase values represent a response to interference in the mobilization of fat, and that the addition of labile methyl groups either in the form of methionine or protein favorably affects both these activities. It should be noted, also, that Salmon (7) has shown with rats that in diets containing 18% or less casein the primary

deficiency, resulting in impaired growth, is labile methyl groups, unless supplements of methionine or choline are added. Our work, in so far as it has gone, is in accord with Salmon's investigations. Further experiments are now in progress to clarify the various relationships between serum alkaline phosphatase, dietary protein, and certain amino acids.

Acknowledgments

The authors wish to express thanks to John Wyeth and Brother (Canada) Limited, for the vitamins and vitamin-free casein used in this investigation.

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THE PREPARATION OF ISINGLASS SOLUTIONS SUITABLE FOR TRANSFUSION PURPOSES[1]

By H. D. Bett

Abstract

The possibilities of producing isinglass solution in sufficiently large quantity to permit adequate clinical trial on a uniform batch have been investigated. A satisfactory process has been developed using fresh frozen hake sounds. Technical details of this preparation are presented in this paper, together with comments on the occurrence and prevention of pyrogens in transfusion fluids. The formation of pyrogens was a continual source of trouble until sterile technique for handling large volumes of material was developed. Technical details are presented, including a method for the removal of pyrogenic substances when, in spite of precautions, these are present. Preparations free from pyrogenic substances can be produced through the use of aseptic technique or by filtration through sterilizing filter pads. In using the latter method it was necessary to limit the quantity of liquid that was filtered through any single pad.

Introduction

During the course of World War II many substances were considered for use as blood substitutes. Gelatin received considerable attention in this respect. Jorns (5) indicated that colloids in isotonic sodium chloride solutions were useful as blood substitutes. Kleinberg and his associates (6) found gelatin effective in the treatment of shock due to trauma. Gordon and associates (2) successfully used gelatin solution to produce recovery in arterial pressure in experimental haemorrhage. Parkins and Lockwood (8) determined that normal dogs tolerated large infusions of gelatin without serious toxic reaction. Gelatin solution was safely administered to normal humans and those in shock by Jacobson and Smyth (4). More recently, Hamilton and associates (3) studied 10 infusion fluids for their effect on haemorrhage in animals. The oxypolygelatin solution reported was superior to physiological saline but less suitable than plasma or 5% albumin solution. McCarthy and Parkins (7) tested the effect of 10 infusion fluids in scald burns in dogs. These authors concluded that the effectiveness of the solutions that afforded the highest effective survival rate could be attributed to sodium salts and water content.

Reports concerning the effectiveness of isinglass solutions as transfusion solutions or blood substitutes are not extensive. Taylor and Waters (11) reported a preparation of isinglass to be useful as a transfusion fluid in experimental haemorrhage in dogs. A further report on this substance was given by Taylor and Moorhouse (10). Pugsley and Farquharson (9) described the use of isinglass solutions in 61 patients. These authors reported that, when properly prepared, isinglass solution appeared to be a safe blood substitute. In a small series of cases of shock the intravenous administration

[1] *Manuscript received April 7, 1948.*

Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont.

of isinglass solution appeared to be of value. The preparation of isinglass from the dried swim bladder of the hake, by the method of Prof. N. B. Taylor is described by Beveridge and Lucas (1). The amino acid content of isinglass is given in the same paper.

In the method used by Taylor, after the development of the isinglass with calcium hydroxide, large volumes of acetone are required. From experience in our laboratory it was found that 25 liters of acetone were required to produce sufficient isinglass for 500 cc. of a 6% solution such as is proposed for treatment. This makes the preparation of reasonably large amounts a matter of some difficulty. It was also found that contamination of the product with pyrogenic substances was readily encountered when dealing with large quantities of hake sounds in the dried state. Since isinglass appeared to have value as a transfusion fluid, a method was sought to lessen these difficulties. Arrangements were made to have sounds promptly frozen on removal from the fish, and shipped under refrigeration conditions. These sounds* arrived in excellent condition and remained so as long as they were kept frozen. Using frozen sounds as starting material, the following method was developed for the production of a large amount of isinglass solution suitable for clinical trial.

Method

In order to reduce the possibility of pyrogen formation to a minimum after the isinglass had been developed, all of the processing equipment was sterilized.

Forty pounds of fresh frozen hake sounds were placed in running tap water in a stainless steel container until the sounds were no longer hard. This required usually about two hours.

The water was drained off and replaced by 30 gal. of saturated calcium hydroxide solution and 50 lb. of ice. The presence of ice assisted in preventing the physical breakup of the sounds and therefore subsequent losses in handling. The sounds were left in this solution for 24 hr. The solution was then replaced with freshly prepared, saturated calcium hydroxide solution and ice. This process was repeated until the sounds had been subjected to six washings. The material was, of course, kept in a refrigerator throughout this period.

The sounds were then washed in tap water and allowed to stand in water for 24 hr. The water was then removed and replaced by 30 gal. of 0.05 *N* hydrochloric acid solution. Two such washes were given at an interval of one hour. Ice was present in all these washes as in the case of the calcium hydroxide treatment. Following this, two washes were made with hydrochloric acid of the same concentration but prepared from freshly distilled, pyrogen-free water. No ice was present. (If freshly distilled water cannot

* *The sounds were procured through the co-operation of Mr. E. B. Richardson of the Maritime National Fish Division of Sea Products Limited, Digby, N.S.*

be obtained in sufficient quantity, water should be distilled and autoclaved immediately until enough is collected. This procedure usually provides pyrogen-free water.)

Upon draining the last hydrochloric acid wash from the sounds they were washed in 30 gal. of 1% sodium chloride solution prepared with freshly distilled, pyrogen-free water. This solution was then removed by draining, leaving a gelatinous mass.

In subsequent parts of the process it is particularly important to avoid contamination. The gelatinous mass was transferred to a clean kettle that had been washed by boiling distilled water in it for at least one hour.

The contents were next heated to 90° C., with constant agitation. This agitation served to stir the mixture and to break up the sounds. On reaching this temperature the mixture was usually homogeneous. Eight pounds of Hy-flo* filter aid were added. The temperature was then lowered to 70° C. A small test sample was removed and filtered through paper** on a Buchner funnel. The sodium chloride content was determined by Volhard's thiocyanate procedure, the pH adjusted with 5 *N* sodium hydroxide solution to 7.2 and total solids determined by evaporation of 5 cc. samples in tared dishes. These three operations should be done in less than one hour. The volume before and after filtering having been noted and the volume of the mixture in the kettle being known, calculations could then be made from the three determinations to determine a dilution sufficient to give final values of about 7% total solids, 0.85% in sodium chloride and pH 7.2. Any diluting was done with freshly distilled water or water that had been freshly distilled and autoclaved. The whole was then filtered through a previously steamed plate-and-frame press (six 1 in. frames, 17 in. square, 1 in. thick required). Number 202 Whatman papers backed by cloth were used.

The filtrate was collected in sterile 10-liter bottles and as soon as possible placed in the autoclave. The temperature was brought up to 120° C. (15 lb. pressure) and held for 45 min. When the steam was shut off the autoclave was allowed to reach 100° C. and normal pressure before being opened. The isinglass solution usually contained a precipitate and had to be filtered before final filling.

The mixture was transferred to a sterile pressure tank and the whole filtered under aseptic conditions at 70° C. through St. No. 3*** filter pad by air pressure. This filtrate was then filled into 400 cc. bottles fitted with plastic screw caps and rubber liners. The bottles were filled to within one inch of the top and the screw caps fitted on and tightened. These bottles were then autoclaved, while sealed, for 30 min. This procedure has, to date, always provided material that has been sterile.

* *Hy-flo procured from Canadian Johns-Manville Company, Toronto.*

** *Whatman 202 was found satisfactory.*

*** *St. No. 3 from Hercules Filter Corporation supplied by General Filtration and Engineering Limited, of Toronto.*

Approximately 60 bottles (400 cc. per bottle) may be prepared from 40 lb. of sounds.

Pyrogens

It has been found possible to prevent the formation of pyrogens by starting with fresh frozen sounds and by keeping exposure to contaminants at a minimum at all times. The sterilization of all material with which the isinglass solution may come in contact, such as rubber hose, extraction kettles, filter papers, and cloths has been found to be essential. In the event that pyrogens develop they can be removed by filtration through a St. No. 3 pad, the pad in this case acting as an adsorbing agent. It has been shown that continued filtration of a pyrogenic solution through the pad will result in pyrogens reappearing in the solution. Two liters of a pyrogenic solution could be filtered, and rendered lower in pyrogen by passage through filter pads approximately 22 cm. in diameter. Table I shows the results obtained in such an experiment when a standard pyrogen test was carried out. Ten cc. of isinglass solution per kilogram were administered to each of five rabbits.

TABLE I

REMOVAL OF PYROGENS BY FILTRATION

	Temperature rise, ° F.	
	Average	Highest
Original solution	1.6	2.5
1st liter through pad	0.3	0.4
6th liter through pad	1.6	1.9
17th liter through pad	1.6	2.4

This process has been repeated on several occasions and whenever pyrogens have been encountered they have been successfully removed by such treatment.

Isinglass solutions have been stored for months without change in pyrogenicity. Two solutions prepared in March and retested in October gave the results shown in Table II.

TABLE II

THE EFFECT OF SEVERAL MONTHS' STORAGE AT 45° F. ON THE PYROGENICITY OF ISINGLASS

		Temperature rise, ° F.	
		Average	Highest
Solution 1			
March	Original solution	0.2	0.3
October	Retest	0.5	1.0
Solution 2			
March	Original solution	0.2	0.7
October	Retest	0.2	0.8

In all, about one hundred pyrogen tests were carried out before the process described was considered suitable for the preparation of isinglass solutions. No disturbances in the rabbits were noticed excepting the hyperthermia in cases where pyrogens were involved.

Discussion

The procuring of fresh frozen sounds was found to be essential if pyrogens were to be kept at a minimum in the preparation of isinglass. The dried sounds previously used for the preparation of isinglass could not be used when the purification by acetone precipitation was omitted. No trouble was found using tap water for the calcium hydroxide solution and the first two hydrochloric acid treatments, but in subsequent parts of the process the use of pyrogen-free water was found essential. Water was freshly distilled into 20-liter bottles, autoclaved at 120° C. for two hours and refrigerated. Water prepared in this manner could be stored until it was required for the washing of various pieces of equipment prior to sterilization by steaming. In the washing of the filter press approximately 40 liters of water were used. During the adjusting of acidity and determination of chlorides and solids content, the isinglass mixture was maintained at 70° C. as an effective means of maintaining sterility and avoiding the formation of pyrogens. In order to avoid changing the pads during the final filtration a press holding 30 pads was used. The filtrate was returned to a kettle in which it was kept at 70° C. and from this was filled, using sterile technique, into the final containers. The 400-cc. bottles were all carefully washed and rinsed with pyrogen-free water, the tops covered with paper, and then autoclaved. It was only by taking such precautions that we were able to repeatedly prepare a solution suitable for use.

Acknowledgments

The author wishes to express his thanks to Dr. N. B. Taylor for having discussed this problem with him personally when this work was undertaken; to Dr. D. M. Young and Mr. G. G. Waters under whose supervision pyrogen testing was carried out; and to Dr. G. A. McVicar for valuable suggestions during the course of this investigation.

Thanks are expressed to Mr. W. H. Brown for his technical assistance in planning and carrying out large scale operations.

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PROTECTION BY KILLED VOLE BACILLUS VACCINE AGAINST EXPERIMENTAL TUBERCULOSIS IN GUINEA PIGS[1]

By J. C. Paterson, D. W. Crombie, and J. C. Coles

Abstract

A vaccine made from vole bacilli killed by ultraviolet light has been shown to confer a significant degree of protection against experimental tuberculosis in guinea pigs. The degree of protection was comparable to that obtained from BCG. The results of this single experiment suggest that further work on this material should be carried out.

Although BCG possesses definite immunizing properties against tuberculosis in man and in experimental animals, it has not been universally accepted by the medical profession. There are those (5) who claim that our knowledge of tuberculosis immunity is not complete enough to justify artificial immunization with living organisms. These same critics suggest that, although inoculation with BCG is apparently not immediately dangerous, organisms may survive for years and may gradually regain virulence. Recently, the U.S. Public Health Service (4) recommended that further research on BCG be carried out before it is licensed commercially, and that attempts be continued to develop a material containing only dead organisms.

Among other materials under development is a vaccine made from the vole bacillus. Wells (7), who discovered this murine type of tubercle bacillus in 1937, found that inoculation with viable organisms conferred a significant amount of protection against experimental tuberculosis in guinea pigs. This finding has been confirmed by others (3, 6,* 2). It is to be noted, however, that living vole bacilli were used in all of these experiments. Preliminary investigations by Crombie and Hambleton, in this laboratory, indicated that *killed* vole bacilli might also confer some degree of protection against experimental tuberculosis in animals. These preliminary studies have been elaborated upon, and the results of a large scale experiment in which the merits of killed vole vaccine and BCG are compared are reported in the present paper.

Material and Method

The series consisted of 150 guinea pigs, weighing from 350 to 550 gm. each, and of approximately an equal sex distribution. The animals were weighed individually, tagged, and segregated. The series was divided into three lots, each lot having the same number of animals, and approximately the same average weight and sex distribution. The animals of Group 1 (50 guinea pigs) were inoculated with killed vole bacillus vaccine, Group 2 (50 guinea

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* *Quoted by Wells* (7).

pigs) with BCG, and Group 3 (50 guinea pigs) were put aside as controls. At an appropriate time, as described later, each animal in the three groups was infected with a large dose of a relatively avirulent strain of human tubercle bacilli. Following this challenge dose, the animals were observed daily, their survival times were noted, they were weighed at weekly intervals, and the sites of inoculation with vole vaccine and BCG were inspected for the presence of abscess formation. These measures were continued until all of the animals had died.

The vaccination with killed vole bacilli was carried out as follows: each of the guinea pigs in Group 1 was inoculated, by subcutaneous injection into the right groin, with two doses of vole bacilli killed by ultraviolet light. The doses were given at an interval of 28 days. The first dose consisted of 4 mgm. and the second of 1 mgm. moist weight of organisms. It was found by experiment that a one-minute exposure of a suspension (1 mgm. per cc.) of vole bacilli, 3 mm. deep in a porcelain plate, to a hot quartz ultraviolet light was sufficient to kill all of the organisms. However, for the actual vaccination, the exposure was increased to two minutes to ensure that all organisms were killed. Samples of the first vaccine were cultured at the time the material was injected, and these cultures were retained for four months when no growth was observed. The second vaccine, which was prepared in an identical fashion, was not cultured until 24 hr. after it was used. During this 24 hr. period the material stood at room temperature. No growth was observed from this second vaccine after four months.

The BCG used for the immunization of guinea pigs in Group 2 was obtained from the University of Montreal, and it was of a standard variety. Two doses of the material, each consisting of 10 mgm. moist weight of organisms, were injected subcutaneously, into the right groin of each animal. The doses were given at an interval of 28 days.

The challenge dose of tubercle bacilli was given by subcutaneous injection into the left groin, 28 days after the guinea pigs in Groups 1 and 2 had received their last dose of either killed vole vaccine or BCG. It consisted of 0.25 mgm. moist weight of a DT strain of human tubercle bacilli.* The actual suspension was prepared so that 1 cc. contained this weight of organisms. Suitable measures were taken to ensure that animals in the three groups received approximately the same amounts of infective material.

On the death of each animal an autopsy was carried out. Blocks of tissue were taken from the inguinal lymph nodes, from each lung at the level of the hilus, and from three different parts of the liver and spleen. The blocks were taken routinely from approximately the same location in each specimen,

** Although most workers have used very small doses (0.000001 mgm.) of a virulent strain of tubercle bacilli (1), we felt that by giving a larger dose of a less virulent strain greater accuracy in dosage could be obtained.*

regardless of the site of the most striking gross lesions. The blocks of tissue were embedded in paraffin, and sections, cut at a thickness of 7μ, were stained with haematoxylin and eosin. The microscopic examination included an assessment of the number and size of individual tubercles in each organ as well as a description of the type of tuberculous process present.

Observations

A number of animals from each group died either before the challenge dose of tubercle bacilli was given or so soon afterward that the deaths could not be attributed, pathologically, to tuberculosis. These deaths from intercurrent infection have been eliminated from the series leaving the following number of animals in each group:

Group 1	(Killed vole vaccine)	44
Group 2	(BCG vaccine)	47
Group 3	(Controls)	45

All of the animals in each group ultimately died following infection with tubercle bacilli. The relative efficacy of the two types of vaccines was assessed by comparing the survival times, the loss in weight, and the extent of tuberculosis at autopsy, of animals in each group.

The average survival time for guinea pigs in the control group was 33 days; that for the killed vole vaccine group was 64 days; and that for the BCG group was 73 days. The average survival time in the two treated groups was, therefore, approximately twice that in the control group. The difference in survival times was particularly impressive at the 58th day of observation when all of the control animals had died while only 49% of the BCG group and 59% of the vole group had succumbed.

The individual survival times for the various animals are listed in Table I, and shown graphically in Fig. 1. They have been subjected to statistical analysis, using the standard error of difference to compare mean survival times, with the following results:

On comparing the control group versus the BCG group, the S.E. diff. = ± 5.96. By calculating Fisher's t value the difference between these means is shown to be highly significant, P being less than 0.01.

Similarly, on comparing the control group versus the vole group, the S.E. diff. = ± 4.17, and the difference is again shown to be highly significant, P being less than 0.01.

On the other hand, on comparing the vole group and the BCG group, the S.E. diff. = ± 6.78, and the difference is not significant, P being between 0.2 and 0.1.

The weight loss, one month after infection with tubercle bacilli, was less marked in the treated animals than in the control animals. At this time, the average weight loss for the control animals was 10.9 gm., for the BCG animals it was 4.9 gm., and for the vole animals it was 3.7 gm.

TABLE I

INDIVIDUAL SURVIVAL TIMES OF GUINEA PIGS AFTER TUBERCULOUS INFECTION

Animal Number	Control group	BCG group	Vole group
1	14 Days	24 Days	30 Days
2	16 "	27 "	39 "
3	16 "	28 "	41 "
4	17 "	29 "	43 "
5	19 "	34 "	46 "
6	19 "	36 "	46 "
7	20 "	38 "	47 "
8	20 "	39 "	48 "
9	21 "	39 "	48 "
10	21 "	40 "	49 "
11	22 "	40 "	49 "
12	23 "	41 "	49 "
13	23 "	41 "	49 "
14	24 "	48 "	49 "
15	25 "	49 "	49 "
16	26 "	49 "	50 "
17	28 "	51 "	50 "
18	28 "	52 "	50 "
19	30 "	52 "	51 "
20	30 "	52 "	54 "
21	30 "	52 "	54 "
22	31 "	55 "	55 "
23	31 "	58 "	55 "
24	31 "	61 "	56 "
25	32 "	69 "	56 "
26	32 "	69 "	58 "
27	33 "	71 "	59 "
28	37 "	71 "	60 "
29	39 "	74 "	61 "
30	39 "	76 "	62 "
31	39 "	76 "	65 "
32	39 "	80 "	66 "
33	40 "	81 "	69 "
34	44 "	82 "	70 "
35	45 "	87 "	85 "
36	46 "	96 "	89 "
37	49 "	98 "	89 "
38	50 "	115 "	89 "
39	50 "	118 "	93 "
40	50 "	122 "	107 "
41	52 "	123 "	110 "
42	52 "	129 "	112 "
43	53 "	130 "	113 "
44	57 "	148 "	151 "
45	58 "	150 "	
46		151 "	
47		178 "	

Inflammatory reaction at the site of vaccination was less pronounced in the vole group than in the BCG group. More than half of the BCG injected animals showed abscesses of the groin, one month after vaccination, and many of these abscesses were large and were complicated by sinus formation. Only 4% of the vole injected animals had local reactions at this time, and in these the lesions were quite trivial.

Pathologically, widespread tuberculous lesions were found in the regional lymph nodes, liver, spleen, and lungs of almost every animal in the series. No significant difference in the number or severity of the lesions in members

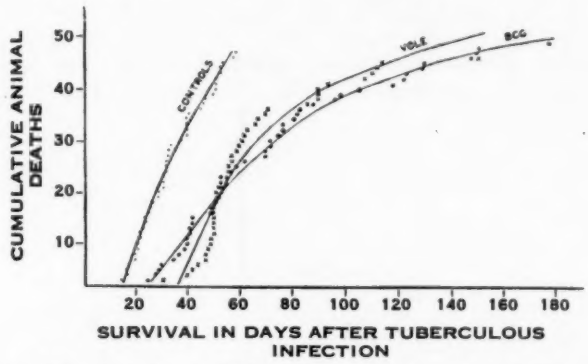

FIG. 1. *The cumulative animal deaths for the control group, the BCG injected group, and the killed vole vaccine group are plotted against the survival time in days.* • = *Controls.* ○ = *BCG.* × = *Vole vaccine.*

of the three groups could be detected; but in this regard it is to be noted that the treated animals lived twice as long as the controls, and the pathological findings are not, therefore, strictly comparable. Caseation of tubercles was more striking in the treated than in the control animals.

Discussion

The results of this large-scale experiment suggest that the vole bacillus, when killed by ultraviolet light and injected into guinea pigs, possesses definite immunizing properties against experimental tuberculosis in this species. The degree of protection afforded was comparable to that obtained from BCG. The efficacy of the killed material was as great as that of vaccines of living vole bacilli, as reported by others (3, 6*, 2).

Although the vole bacillus is said to be nonpathogenic to man (7), the advantages of a vaccine made from dead organisms only are obvious. It is recommended, therefore, that further experimental work be carried out on killed vole bacillus vaccine as an alternative to BCG, and to living vole vaccine, for human immunization. One refinement on our procedure would be to use either a smaller dose of infecting tubercle bacilli or a less virulent strain of organisms, and thus avoid the overwhelming infections that we achieved.

Acknowledgments

We are indebted to Miss Jean Millar for the statistical analyses reported in this paper.

* *Quoted by Wells* (7).

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THE MUCOPOLYSACCHARIDES OF HUMAN SKIN[1]

By R. H. Pearce and E. M. Watson

Abstract

The mean values and standard deviations for the concentrations of the hyaluronic acid and chondroitin sulphuric acid fractions isolated from 11 specimens of human skin were 24.5 ± 5.7 and 26.2 ± 4.7 mgm. per 100 gm. of fresh tissue, respectively.

The individual differences in the concentrations of these substances were masked by the technical errors. The small variations in moisture and ash content of the skin did not contribute appreciably to the uncertainty of the concentration values. The purity of the products, determined by their amino sugar content, approximated 60%, which value compared favorably with those obtained by other workers.

The content of hexosamine and uronic acid identified both fractions as acid mucopolysaccharides. The release of reducing substances, including N-acetyl hexosamine, from the hyaluronic acid fraction by hyaluronidase paralleled the action of the enzyme upon hyaluronate from umbilical cord. The chondroitin sulphuric acid fraction, which was relatively resistant to the action of hyaluronidase, was assumed to be identical with the chondroitin sulphatelike substance that Meyer and Chaffee isolated from pig skin.

Introduction

The connective tissue, including the so-called ground substance, evidently participates in certain vital processes, in addition to exercising its wholly physical role in the provision of cohesion, support, and protection for the various cellular structures of the organism. Consequently, an active interest in the physiological and pathological significance of this tissue is developing (10). The present investigation is concerned with the mucopolysaccharides that are characteristic constituents of the connective tissue. The methods of analysis relating to these substances that have been evolved may facilitate investigations of their metabolism.

The mucoproteins, which are commonly referred to as mucins or mucoids, consist of saltlike combinations of a protein cation with a carbohydrate anion, the degree of their association being dependent upon the pH of the dispersing medium. The characteristic hydrophilic properties of the solutions from which such materials are separable appear to be related largely to the nature of the carbohydrate moiety. Therefore, the concentration of the so-called mucopolysaccharides in a tissue provides the best index of its mucinous nature.

As early as 1860, Rollett (18) reported that a constituent of a limewater extract of skin was precipitated with acetic acid and, because of the loss of tensile strength accompanying the extraction process, concluded that this

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mucoprotein served as a cement substance. Van Lier (19) isolated a protein-free polysaccharide that contained sulphur, nitrogen, and either a pentose or a uronic acid from the skins of cattle, horses, dogs, and human beings. The presence of amino sugar in the mucoprotein derived from cow hide was demonstrated by Grassman *et al.* (5) and their data, as interpreted by Meyer and Chaffee (11), indicated also the presence of uronic acid.

Both Claude (3) and Chain and Duthie (2) isolated from rabbit skin a substance, the viscosity of which was reduced by the action of hyaluronidase. The latter workers noted also the release of N-acetyl hexosamine during the enzymatic hydrolysis. Meyer and Chaffee (11) isolated from pig skin two mucopolysaccharide fractions, one of which was identified as hyaluronic acid by the molecular ratio of nitrogen, glucosamine, glucuronic acid, and acetyl to titrable acid groups; by the optical rotation; and by the kinetics of its enzymatic hydrolysis. The other fraction contained nitrogen, galactosamine, glucuronic acid, and acetyl in the proportions found in chondroitin sulphuric acid but the specific rotation did not correspond with that of the latter compound. Bensley (1) showed by histological means the presence of a viscous mucinlike substance between the fibers of the corium of the guinea pig and Reuter (17) observed substances exhibiting the staining properties of the mucins in human skin.

Methods

The Estimation of the Cutaneous Mucopolysaccharides

Since no record of a critical investigation of the methods for the isolation of the mucopolysaccharides could be found, adequate criteria were not available to serve as a guide in the choice of a technique for the separation of these materials. Therefore, after preliminary trials of several procedures described by various writers, the method devised by Meyer and Chaffee (11) for the isolation of the mucopolysaccharides from pig skin was chosen as a basis for our work because of the high yield and excellent appearance of the products. This method was modified to provide a measure of the concentration of the mucopolysaccharides in human cutaneous tissue. A flow sheet illustrating the process is shown in Fig. 1.

The specimens of skin were obtained from legs that had been amputated surgically. The skin was carefully dissected from the limb as soon as possible following the operation with the inclusion of a minimum of subcutaneous fat. Areas exhibiting any gross pathological lesions were excluded from the sample. If the analysis could not be performed immediately, the tissue was stored in acetone at 4° C. Each specimen was ground in a Latapie mill using a known and approximately equal volume of distilled water as a lubricant.

An equal portion of *N* sodium hydroxide was added to the homogeneous paste. The resulting mixture was shaken three or four times daily during its incubation at 37.5° C. At the termination of the 48 hr. extraction period, the tissue had disintegrated almost completely yielding a slightly opalescent

reddish-brown solution. Glacial acetic acid was added dropwise, with stirring, until the pH reached 7.0. The mixture was then clarified with Hyflo Supercel and vacuum filtration.

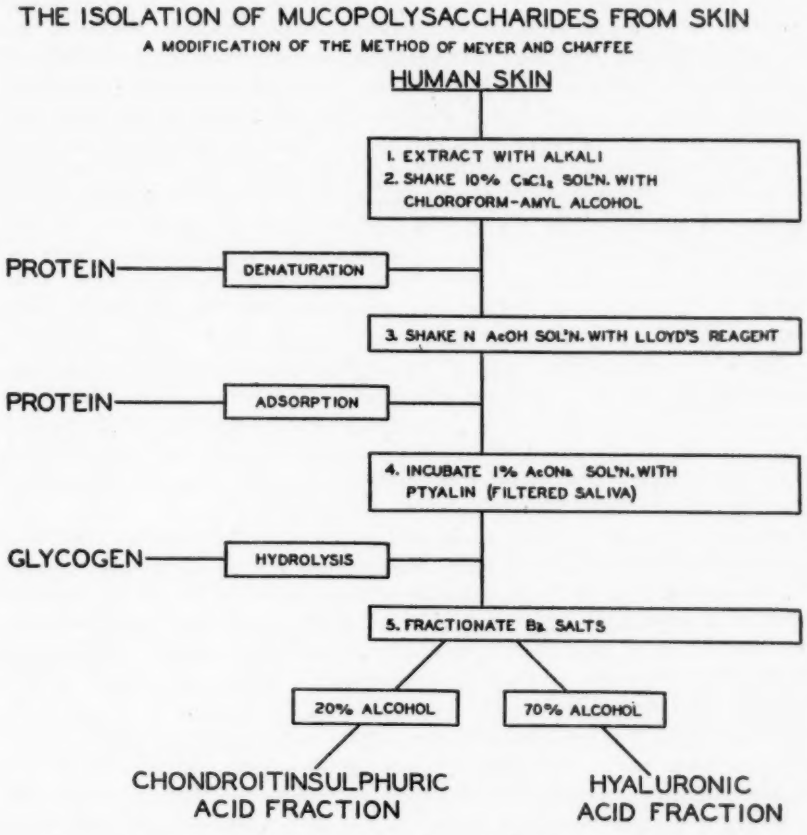

FIG. 1. *A flow diagram for the isolation of the mucopolysaccharides from the skin.*

A boiling water bath was used to concentrate the extract until it became quite viscid; then the mucoprotein was precipitated by transferring the fluid with a minimal amount of wash water to two volumes of a mixture of 95% ethanol containing 1% (w/v) of potassium acetate and 1% (v/v) of glacial acetic acid. This precipitation mixture was used in all the separations described below.

The acid minimized the dissociation of the mucopolysaccharides, while the acetate probably neutralized the micellar charge. These actions, together with the dehydrating effect of the alcohol, produced a dense, easily separable

product. Although the flocculation was apparently complete after the mixture had stood for two to three hours at 4° C., the precipitate could be obtained more readily when it had been left in the refrigerator over night.

The pale brown, gelatinous residue that separated upon centrifugation was dispersed in 50 ml. of 10% (w/v) calcium chloride solution. Following the addition of 10 ml. of a 4 : 1 mixture of chloroform and amyl alcohol and shaking for 48 hr., the denatured protein gel was centrifuged down and the slightly turbid supernatant was poured into 100 ml. of the precipitation mixture. In order to remove the last traces of protein, the white fibrinlike precipitate that formed was centrifuged off and dispersed in 20 ml. of distilled water. The latter was made one normal with respect to acetic acid by the addition of 1.2 ml. of the glacial acid. About 1 gm. of Lloyd's reagent was added and, after a thorough mixing, the suspension was centrifuged and the centrifugate was poured into 60 ml. of the precipitation mixture. The residue was washed with 10 ml. of *N* acetic acid and this centrifugate was also added to the precipitation mixture.

The white asbestoslike fibers of the separated mucopolysaccharides were dissolved in 10 ml. of a 1% (w/v) solution of sodium acetate to which 0.4 ml. of filtered saliva and five drops of toluene were added. The tightly corked tube was incubated subsequently for six or seven hours; then its contents were poured into 20 ml. of the precipitation mixture. This step eliminated any glycogen that may have been present.

After being dissolved in 10 ml. of 10% (w/v) barium acetate solution, the purified mucopolysaccharides were fractionated in the following manner. The barium acid chondroitin sulphate was precipitated by the addition of 2 ml. of 95% ethanol, which brought the alcohol concentration up to 20%, and the container was placed in a refrigerator over night. The barium hyaluronate was separated from the centrifugate by adding the latter to 32 ml. of the precipitation mixture.

The resultant barium salts of the mucopolysaccharides were separated by means of a centrifuge and dissolved in 10 ml. of distilled water. The chondroitin sulphate fraction usually gave a turbid solution, whereas the hyaluronate fraction was water-clear. A 1 or 2 ml. aliquot was removed with a pipette for subsequent analytical investigation and the remainder of the fluid was poured into 20 ml. of the precipitation mixture. The reprecipitated mucopolysaccharide was washed by centrifugation with 95% ethanol and transferred to a prepared filter paper that had been dried over night in an oven at 110° C. and kept in a desiccator over anhydrous calcium chloride prior to being weighed by the method of short swings. The residue on the paper was thoroughly washed three or four times with both ethanol and ether. After air-drying, the paper was placed *in vacuo* over anhydrous calcium chloride over night. The air was admitted to the desiccator through concentrated sulphuric acid and the paper containing the sample was weighed as before. The weighing was repeated at 24-hr. intervals until the readings were constant within 5%.

The weight of the mucopolysaccharide was corrected for the removal of the aliquot and for its theoretical barium content. The concentration of the acid mucopolysaccharide was expressed in relation to the fresh weight of the skin.

The Moisture and Ash Content of the Skin

Samples of the homogenized skin were dried *in vacuo* over anhydrous calcium chloride and phosphorus pentoxide until the weight remained constant within 0.3%. The dried tissue was charred carefully over a Bunsen burner and ignited at 550° C. in an electric oven for several hours until its loss in weight, after cooling in a desiccator, was less than 4%. The weight of the skin in the sample was calculable from the proportions of water and tissue used in the process of grinding.

The Determination of Hexosamine

First Procedure.—To a suitable aliquot of a solution containing the mucopolysaccharide, concentrated hydrochloric acid was added to bring its concentration to 4 *N*. The ampoule containing the mixture was evacuated and the polysaccharide was hydrolyzed by placing it in an oven at 110° C. for eight hours. The excess acid was 98 to 99% neutralized by slowly titrating the hydrolyzate with 7.5 *N* sodium hydroxide to a pH between 2.5 and 5.0, with constant stirring. The contents of the ampoule were then transferred to a 10 ml. volumetric flask, diluted to volume, and kept at 4° C. until ready for analysis. While this technique avoided the liberal dilution associated with the hydrolytic procedure employed by Palmer *et al.* (16), a considerable error was introduced as a result of the difficulties of the microtitration and the destruction of hexosamine by localized excesses of alkali.

The amino sugar content of the hydrolyzate was determined by the method of Palmer *et al.* (16), modified somewhat for use with the Cenco-Sheard-Sanford Photelometer and with the employment of an Evelyn 565 mμ light filter.

Second Procedure.—The method of Palmer *et al.* (16) was used for the hydrolysis and the color development but with the additional sensitivity provided by the Beckman spectrophotometer. The optical densities were read at 530 mμ.

The Determination of Acetyl Hexosamine

The method of Morgan and Elson (15) for the determination of acetamidohexoses was adapted for use with the Cenco-Sheard-Sanford Photelometer. An Evelyn 565 mμ light filter was employed.

The Determination of Uronic Acid

The technique of Maughan, Evelyn, and Browne (9) was modified so that the light transmission could be read with a Cenco-Sheard-Sanford Photelometer at 565 mμ.

The Determination of Reducing Substances

First Procedure.—The method of Folin and Malmros (4) was adapted for use with the Klett-Summerson photoelectric colorimeter. The transmission band of the 660 mμ light filter coincided more closely with the wave length of maximal adsorption and it was consequently more suitable for this analysis than the 540 mμ filter recommended by the manufacturer (7). The gum ghatti in the color reagent was unnecessary for stabilizing the ferric ferrocyanide suspension at the concentrations of glucose that were being measured. The light transmission reached a minimum 20 min. after the addition of the color reagent and was read at this time.

Second Procedure.—The sensitivity of the Folin-Malmros (4) method was increased sufficiently for use with the Cenco-Sheard-Sanford Photelometer by avoiding the dilution prior to the addition of the color reagent. A Klett 540 mμ filter was used.

The Colorimetric Technique

A standard solution of the substance being determined and a blank were run with each batch of test solutions so that a calibration curve could be constructed for each group of analyses. Every procedure was investigated carefully to determine the concentration range in which Beer's law applied, as well as the expected error.

TABLE I

THE CONCENTRATION OF THE MUCOPOLYSACCHARIDE FRACTIONS IN HUMAN SKIN

Specimen No.	Wt. of fresh skin, gm.	Sex	Age, yr.	Hyaluronic acid,* mgm./100 gm.	Chondroitin sulphuric acid,* mgm./100 gm.	H/C ratio
86	232	M	73	26.0	24.6	1.06
87	220	M	79	29.4	24.0	1.23
88	121	M	64	34.6	31.0	1.12
89	131	F	62	14.3	27.4	0.52
90	121	F	70	19.8	24.0	0.82
91	118	F	70	26.2	29.0	0.90
92	236	M	71	18.8	18.8	1.00
93**	255	—	—	21.6	31.8	0.68
212	124	F	77	23.0	29.2	0.79
226	184	M	69	27.7	18.2	1.52
231	143	M	86	27.9	30.5	0.92
Mean				24.5	26.2	0.96
Standard deviation				5.7	4.7	0.27
Standard error				1.7	1.4	0.08

* *The mucopolysaccharides were isolated and weighed as their barium salts. The yields were corrected for the removal of an aliquot for analysis and the concentrations were expressed as the amount of the free acid in the fresh skin.*

** *This specimen of skin was obtained from an arm.*

Results

The Concentration of the Cutaneous Mucopolysaccharides

Samples of skin obtained from 10 legs amputated above the knees because of gangrene of the feet were analyzed in the manner described above. A specimen secured from an arm was also included. The concentrations of the cutaneous mucopolysaccharides in these specimens are given in Table I. The relative frequency at which the various concentrations of each substance were observed has been demonstrated by grouping the data under two sets of class intervals in order to provide a better over-all picture of the distribution than that obtainable from a simple histogram. The results for the hyaluronic and chondroitin sulphuric acid fractions are presented graphically in Figs. 2 and 3, respectively.

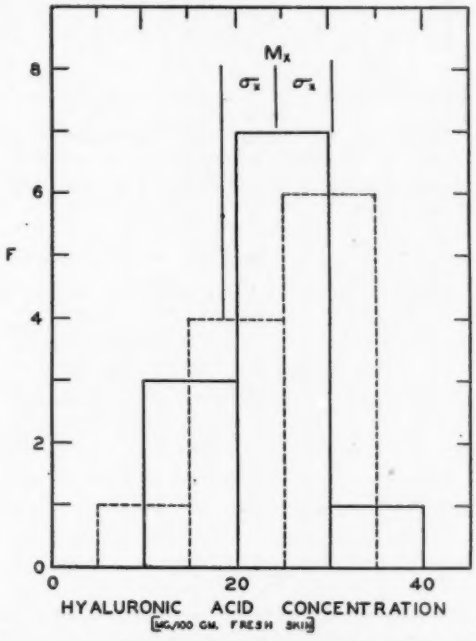

FIG. 2. *The frequency distribution of the hyaluronic acid concentrations in human skin. The data are grouped under two sets of class intervals.*

The Relationship of Age and Sex to the Cutaneous Mucopolysaccharides

The observed values were surveyed with a view to deciding whether or not the sex or the age of the individuals bore any relationship to the concentration of the cutaneous mucopolysaccharides. The results of the analyses of the skin from the legs of six men and four women were considered. The mean concentrations for each of these groups of individuals, with the associated standard errors, may be seen in Table II.

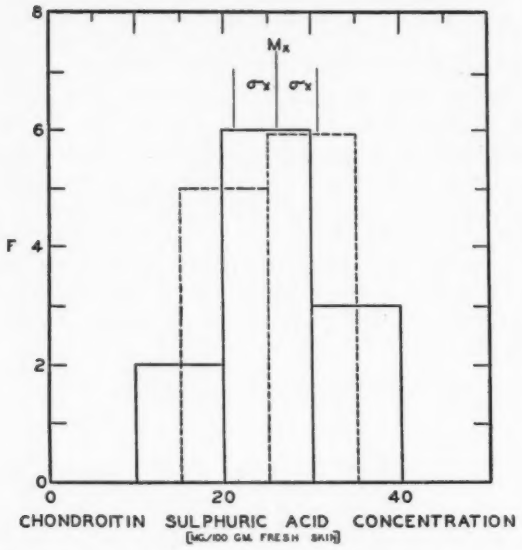

FIG. 3. *The frequency distribution of the chondroitin sulphuric acid concentrations in human skin. The data are grouped under two sets of class intervals.*

TABLE II

THE RELATIONSHIP OF THE SEX OF THE INDIVIDUAL TO THE CUTANEOUS MUCOPOLYSACCHARIDE CONCENTRATIONS*

Sex	No. of samples	Hyaluronic acid, mgm./100 gm.	Chondroitin sulphuric acid, mgm./100 gm.	H/C ratio
Male	6	27.4 ± 2.1	24.5 ± 2.2	1.14 ± 0.09
Female	4	20.8 ± 2.5	27.4 ± 1.2	0.76 ± 0.08
	P	0.07	0.36	0.019

* *Calculated from the data presented in Table I. The means are given with their standard errors.*

The Reproducibility of the Method

The limited amount of normal skin that was obtainable from any single specimen did not permit multiple analyses with the provision of yields sufficient for accurate weighing. Consequently, the error inherent in the method was assessed by analyzing the material derived from the grossly thickened skin from an amputated myxoedematous leg. This skin was treated in the same manner as the control specimens of skin referred to above except that, after

the tissue was ground, it was further diluted with an equal volume of water so that the mucopolysaccharide concentration would be of approximately the same order as that occurring in the control specimens. Three samples of the same specimen were carried through the isolation processes individually. The data shown in Table III were obtained.

TABLE III

THE REPRODUCIBILITY OF THE YIELDS OF MUCOPOLYSACCHARIDES

Specimen No.*	Hyaluronic acid**, mgm./100 gm.	Chondroitin sulphuric acid**, mgm./100 gm.
229–2	57.1	47.2
229–1	60.9	48.4
229–3	72.9	50.6
Mean	63.6	48.7
S.d.	8.2	1.7

** The skin from a patient with pretibial myxoedema was analyzed.*
*** The data were calculated in the same manner as those in Table I.*

Furthermore, two groups of control specimens that were analyzed 17 months apart yielded almost identical values, thus providing an additional indication of the reproducibility of the analytical technique.

The Moisture and Ash Content of the Skin

Since the concentration of the mucopolysaccharides in the skin has been expressed in terms of the fresh weight of the tissue, one might suspect that a large proportion of the variability of the results are explainable by differences in the amounts of water and inorganic materials in the skin of different individuals. The extent to which such factors influenced the results was judged by determining the moisture and ash content of four specimens of skin that were undergoing analysis. The observed values are given in Table IV.

TABLE IV

THE MOISTURE AND ASH CONTENT OF HUMAN SKIN

Specimen No.	Moisture, %	Ash, %
230	61.8	1.08
225	60.8	0.72
231	61.4	0.36
226	60.2	0.78
Mean	61.0	0.74
S.e.	0.4	0.15

The Purity of the Products

The determination of the amino sugar content of the mucopolysaccharides provided a simple measure of the purity of the isolated fractions, since hexosamine is a characteristic component of the molecule and its theoretical concentration can be calculated. The results of such a determination are recorded in Table V. The low values that were obtained were attributable in part at

TABLE V

THE PROPERTIES OF THE MUCOPOLYSACCHARIDE PREPARATIONS

Property studied	Hyaluronate		No. of samples	Chondroitin sulphate		No. of samples	*P*
	Mean	S.d.		Mean	S.d.		
Hexosamine content as % of theory*							
1st series	60	16	11	60	13	10	—
2nd series	79	10	4	54	5	4	—
Enzymatic hydrolysis as % of theory							
1st series** (*a*)	69	17	11	−1	3	11	<0.001
(*b*)	145	50	11	−2	6	10	<0.001
2nd series*** (*a*)	11.5	1.8	3	3.7	1.1	3	<0.001
(*b*)	15.2	3.6	3	7.4	1.9	3	0.011

** Determined by comparing the estimated hexosamine content with the theoretical value.*

*** Estimated by the release of N-acetyl hexosamine by hyaluronidase at pH 6.0 and calculated (a) from the weight and (b) from the observed hexosamine content of the preparations.*

**** As indicated by the release of reducing substances through hyaluronidase action at pH 6.0 in 0.3 M sodium chloride and expressed as for the first series.*

least to the destruction of the hexosamine during the neutralization of the hydrolyzate. A proportion of the error in the estimate of purity doubtless could be ascribed to the uncertainties in the values for both the concentration of hexosamine and the weight of the sample.

Another series of preparations was analyzed by the more accurate "second procedure" described above. The values that were obtained (see Table V) indicated that the products contained some contaminating material.

As a further check of the general efficacy of the isolation technique, a specimen of hyaluronic acid and of calcium acid chondroitin sulphate, each prepared in this laboratory, were subjected to analysis and the results compared with those that Meyer (10, 12, 13) had obtained under similar conditions.

The Response of the Mucopolysaccharide Preparations to Hyaluronidase

A diluted portion of the aliquot retained for analysis was added to an equal volume of 0.1 *M* acetate buffer, pH 6.0, containing 1 mgm. of hyaluronidase, i.e. approximately seven turbidity reducing units, per ml. The mixture was

divided into two parts, in one of which the enzyme was inactivated by raising the temperature to 100° C. for 30 min. The other was incubated for 24 hr. at 37.5° C. in the presence of toluene and the enzyme was destroyed subsequently by heating. Aliquots were removed from each of these mixtures and their content of free N-acetyl hexosamine and uronic acid was estimated.

The theoretical amount of N-acetyl hexosamine present was calculated on the basis of the yield of the mucopolysaccharide that had been isolated, using the molecular weights of the moieties deduced to be present in the molecule. By comparing this theoretical amount of acetyl hexosamine with the amount released by the enzyme, an estimate of the degree of hydrolysis was obtained (see Table V). The errors both in the estimated concentration of acetyl hexosamine and in the weight of the sample accounted for a large part of the error in the observed values. The highly significant difference in the responses of the hyaluronate and chondroitin sulphate fractions is indicated by a '*t*' value of 12.7.

Since these mucopolysaccharides had been found to be quite impure, a better estimate of the degree of enzymatic hydrolysis was sought by correcting the value based upon the weight of the sample for the hexosamine content of the mucopolysaccharide preparations. The results of these computations are given in Table V. The considerable error in this corrected degree of hydrolysis originated from the errors involved in the estimates of both the purity and the degree of hydrolysis.

The results of the analyses for uronic acid in the same solutions could not be interpreted on a quantitative basis. However, the color that was produced in each test provided qualitative evidence of the presence of uronic acid in the molecule.

In view of the anomalous results that were obtained with the above technique, the extent of enzymatic hydrolysis of a second series of preparations was estimated by a slightly different procedure. The strength of the acetate buffer was doubled and sodium chloride was added to a concentration of 0.6 *M* in order to eliminate the inhibition of the enzyme, which is known to occur in the absence of these ions. The quantity of glucose equivalent to the reducing substances that were released by the enzyme was determined by the "first procedure." The amount equivalent to a complete hydrolysis was calculated from the data of Meyer, Smyth, and Dawson (14) and the degree of hydrolysis computed therefrom. This value was corrected further for the impurities present as determined by analyses for hexosamine. These data may be found in Table V.

The Kinetics of the Enzymatic Hydrolysis

A specimen of the cutaneous hyaluronate fraction and a specimen of hyaluronic acid prepared by the same procedure from umbilical cord were dissolved in McIlvane's buffer, pH 6.0, at a concentration that approximated 0.4 mgm. per ml. The quantity of mucopolysaccharide that was present in each solution was estimated by its content of hexosamine using the "first

procedure." Samples from each of these solutions were added to equal portions of a series of diluted solutions of hyaluronidase in 0.1 *M* acetate buffer, pH 6.0. After incubation for 2 and 20 hr. at 37.5° C., aliquots were removed from the mixture for the estimation of reducing substances by the "second procedure." The degree of hydrolysis was calculated from the proportion of the total theoretical amount of reducing substances that had been released. The results of these calculations are presented in Fig. 4.

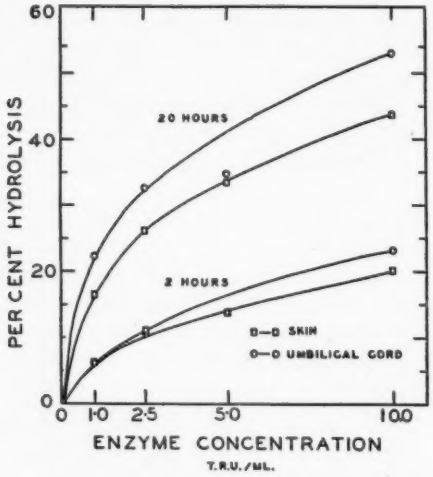

FIG. 4. *The kinetics of the hydrolysis of the cutaneous mucopolysaccharide and of the hyaluronate from umbilical cord at pH 6.0 and 37.5° C.*

Discussion

Biochemical studies of the mucopolysaccharides have been hampered by a lack of appropriate analytical methods. Therefore, the results of an investigation of this kind can be interpreted only in the light of the limitations thus imposed.

In so far as may be judged from the data presented, the proposed modification of the method of Meyer and Chaffee (11) for the isolation of the mucopolysaccharides from pig skin provides an index of the concentration of these components in human skin.

While the products obtained by this procedure were not pure, the degree of their contamination was uniform and therefore did not contribute an appreciable error to the results. The amount of impurities detected was not extraordinary considering the known colloidal properties of the mucopolysaccharides and the complex character of the original material. Indeed, the increase in the concentration of the substances sought from 0.03% in the fresh skin to 60% in the isolated product provided a favorable indication of the efficacy

of the isolation process. The degrees of purity of the mucopolysaccharides prepared in this laboratory were almost identical with those of Meyer's preparations (10, 12, 13).

The standard deviation observed in triple analyses of a single specimen of skin was greater than that found in a series of analyses. Consequently, the error connected with the analytical technique *per se* must have masked any individual differences in concentration. Since the ratio of the yields of the two fractions was more constant than the yields themselves, this error must have originated principally in the separation processes rather than in the fractionation procedure. The greater consistency of the yields of the chondroitin sulphate fraction, as compared with those of the hyaluronate fraction, probably resulted from the more specific means for its separation.

The remarkably constant moisture and ash content of the skin clearly indicated that the expression of the results in terms of the fresh weight of the skin did not diminish appreciably their reproducibility.

While the exact nature of the products could not be established definitely, the presence of N-acetyl hexosamine and uronic acid pointed to these substances being acid mucopolysaccharides. The concentration of hexosamine was sufficiently close to theory to support this suggestion.

The one fraction closely resembled hyaluronic acid as regards the extensive release, by the action of hyaluronidase, of reducing substances, including a product that gave the color reaction of N-acetyl hexosamine. The kinetics of this hydrolysis were paralleled by those of an hyaluronate preparation from umbilical cord. The high value for the extent of the apparent hydrolysis as determined by the release of N-acetyl hexosamine was, at first, extremely puzzling. However, since the completion of these analyses, Humphrey (6) has shown that an intermediate breakdown product of hyaluronic acid gives an intense color identical with that produced by N-acetyl hexosamine, so that an apparent N-acetyl hexosamine content approximately double the expected value is possible. The most likely explanation for this phenomenon is that the N-acetyl hexosamine is transformed by the analytical procedure only partially into the supposed cyclic condensation product responsible for the color development, whereas this condensation product is released as such from the hyaluronic acid upon enzymatic hydrolysis. No substance other than hyaluronic acid is known to possess this property.

The other fraction could not be identified as accurately as that referred to above. However, the observed data coupled with the knowledge that Meyer and Chaffee (11) isolated hyaluronic acid and a chondroitin sulphuric acid-like substance from pig skin have led to the tentative opinion that the two fractions in question were identical with those obtained by these investigators.

The concentrations of the mucopolysaccharides in the skin from human legs, as determined by this technique, may, of course, only approximate the true values since there is no proof that their separation was complete.

The distribution of the observed values was accounted for entirely by the deficiencies of the analytical procedures, leading to the conclusion that the individual differences in the concentrations must have been relatively small. The frequency distribution of the observed concentrations was sufficiently close to normal to warrant statistical comparisons based upon the Gaussian curve of error.

While the age of the individual bore no apparent relationship to the cutaneous mucopolysaccharide concentrations, there seemed to be less hyaluronic acid in the skin of the females that were examined than in that of the males. This difference was masked by the variability of the observed concentrations but became apparent when the ratio of the two components was considered. This observation is in accord with the lesser intracutaneous resistance of female rabbits as compared with males (8).

Summary

1. An adaptation of a method described by Meyer and Chaffee (11) for the isolation of the mucopolysaccharides from pig skin was utilized for the quantitative estimation of the mucopolysaccharides in human skin.

2. By this means, the concentration of hyaluronic acid and of another acid mucopolysaccharide, probably chondroitin sulphuric acid, in 11 samples of normal skin was found to be 24.5 ± 5.7 and 26.2 ± 4.7 mgm. per 100 gm. of fresh skin, respectively. (These are standard deviations.)

3. Less hyaluronic acid was observed in the skin of women than in that of men.

Acknowledgments

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